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(54) Title: CLONING AND USES OF THE GENETIC LOCUS BCL-6

(57) Abstract

This invention provides methods or regulating BCL-6 levels in cells and a method of treating a subject with lymphoma.

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CLONING AND USES OF THE GENETIC LOCUS bcl-6

This application claims priority of U.S Serial No. 09/107,058, filed June 30, 1998, the content of which is hereby incorporated by reference into the subject application.

The invention disclosed herein was made with Government support under NIH Grant Nos. CA-44029, CA-34775, CA-08748 and CA-37295 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Throughout this application various references are referred to within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each Experimental Detail Section.

Non-random chromosomal abnormalities are found in up to 90% of patients with non-Hodgkin's lymphoma (NHL) and have been shown to play an important role in lymphomagenesis by activating proto-oncogenes (1). Some of these translocations, which are associated with specific histologic subsets of NHL, have been characterized at the molecular level. In the t(8;14), t(8;22), and t(2;8) translocations associated with Burkitt Lymphoma, L_3-type acute lymphoblastic leukemia and AIDS-associated non-Hodgkin lymphoma (NHL), a known proto-oncogene, c-<u>myc</u>, was found juxtaposed to the immunoglobulin (Ig) loci (2,3). In the t(14;18) translocation, which is implicated in follicular-type NHL, molecular analysis of the sequences linked to the Ig locus led to the identification of a novel proto-oncogene, <u>bcl-2</u> (4-6). The t(11;14)(q13;q32), mainly associated with "mantle zone" lymphoma, appears to involve

the juxtaposition of the Ig heavy-chain locus with the bcl-1 locus, the site of the candidate proto-oncogene PRAD-1/cyclin D1 (7,8). These well characterized chromosome translocations are associated, however, with only a fraction of NHL cases, while a number of other recurrent translocations remain to be characterized for their genetic components.

One important example of such cytogenetic abnormalities is represented by various alterations affecting band 3q27. This region is involved in translocations with various chromosomal sites including, but not limited, to those carrying the Ig heavy-(14q32) or light- (2p12, 22q11) chain loci (9,10). Overall, 3q27 breakpoints are detectable in 7-12% of B-cell NHL cases by cytogenetic analysis, with t(3;22)(q27;q11) being the most frequent type detectable in 4-5% of NHL (9). The clinicopathologic relevance of 3q27 breakpoints is underscored by its consistent association with diffuse-type NHL, a frequent and clinical aggressive subtype for which no specific molecular lesion has yet been identified (9).

The recurrence of 3q27 breakpoints in NHL has prompted a search for the corresponding proto-oncogene. This invention discloses the cloning of clustered 3q27 breakpoints from two NHL cases carrying t(3;14)(q27;q32) translocations and the identification of genomic rearrangements within the same breakpoint region in additional NHL cases carrying translocations involving 3q27. Within the same region, a transcriptional unit has been identified, which represents the candidate proto-oncogene (bcl-6) associated with 3q27 translocations in B-NHL.

SUMMARY OF THE INVENTION

This invention provides an isolated vertebrate nucleic acid molecule of bcl-6 locus. This invention provides an isolated vertebrate DNA molecule of bcl-6 locus. This invention provides an isolated vertebrate cDNA molecule of bcl-6. This invention provides an isolated genomic DNA molecule of bcl-6. This invention provides an isolated vertebrate RNA molecule of bcl-6. This invention provides an isolated human nucleic acid molecule of bcl-6 locus.

In addition, this invention provides a nucleic acid molecule comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of the nucleic acid molecule of bcl-6.

In addition, this invention provides an isolated vertebrate DNA molecule of bcl-6 operatively linked to a promoter of RNA transcription. This invention provides a vector which comprises the isolated vertebrate DNA molecule of bcl-6.

In addition, this invention provides the above vector, wherein the isolated nucleic acid molecule is linked to a plasmid.

In addition, this invention provides a host vector system for the production of a polypeptide encoded by bcl-6 locus, which comprises the above vector in a suitable host.

In addition, this invention provides a method of producing a polypeptide encoded by bcl-6 locus, which comprises growing the above host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

In addition, this invention provides a polypeptide encoded by the isolated vertebrate nucleic acid molecule of bcl-6

locus. Further, this invention provides an antibody capable of binding to polypeptide encoded by bcl-6 locus.

In addition, this invention provides an antagonist capable of blocking the expression of the polypeptide encoded by bcl-6.

In addition, this invention provides an antisense molecule capable of hybridizing to the nucleic acid molecule of bcl-6.

In addition, this invention provides an assay for non-Hodgkin's lymphoma, a method for screening putative therapeutic agents for treatment of non-Hodgkin's lymphoma and a method for diagnosing B-cell lymphoma.

Finally, this invention provides a method of treating a subject with non-Hodgkin's lymphoma.

This invention further provides a method of degrading BCL-6 in cells comprising administering a molecule which induces phosphorylation of BCL-6 and thereby induces BCL-6 degradation.

This invention provides a method of treating a subject with lymphoma which comprises administering an effective amount of a pharmaceutical composition comprising a molecule which induces phosphorylation of BCL-6 protein so as to induce degradation of BCL-6 and a pharmaceutically acceptable carrier, thereby treating the subject with lymphoma.

This invention provides a method of deceasing BCL-6 levels in cells comprising administering a compound which interferes with transcription of bcl-6 and thereby prevents expression of BCL-6 protein so as to thereby deceasing BCL-6 levels in the cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Immunoglobulin gene rearrangement analysis of KC1445 and SM1444 DNA. DNA extracted from the cell lines U937 (monocytic leukemia) and SK-N-MC (neuroblastoma) were used as controls for non-rearranged, germ-line Ig genes. In the left panel, the arrow on the left points to the rearranged J_H fragment which does not contain C_μ sequences in KC1445 DNA, while the two arrows on the right point to the two distinct fragments containing J_H or C_μ sequences in SM1444 DNA.

Figure 2: Molecular cloning of the chromosomal breakpoints from two NHL cases with t(3;14). Illustrated are the maps of two representative phage clones spanning the breakpoint regions in case SM1444 (SM-71) and KC1445 (KC-51). Chromosome 14 portions of the phage inserts are indicated by a solid line with hatched and black boxes representing switch sequences and C_μ exons, respectively. Vertical arrows point to the junctions of chromosome 3 and 14 sequences. The probes used for Southern (Figure 4) and Northern (Figure 5) analysis are illustrated below the SM-71 map. Restriction enzyme sites are indicated as: B=*Bam*HI; H=*Hind*III; R=*Eco*RI; G=*Bbl*II; S=*sac*I.

Figure 3: Localization of phage SM-71 sequences to chromosomes 3 and 14 by fluorescence *in situ* hybridization. Consistent hybridization signals at 3q27 (arrow in panel A) and 14q32 (arrow in panel B) demonstrated that the insert is derived from the translocation junction.

Figures 4A-4C: Southern blot hybridization analysis of bcl-6 rearrangements in NHL carrying 3q27 breakpoints. The probes used are illustrated in Figure 2. U937 and SK-N-MC DNAs are used as germ-line controls since their hybridization pattern was identical to the one observed in a panel of 19 control DNAs tested. The detected cytogenetic abnormalities affecting 3q27 in each case are: KC1445: t(3;14)(q27;q32); SM1444: t(3;14)(q27;q32); TF1403: t(3;14)(q27;q32); LD1411: t(3,14)(q27;q32); EM352: t(3;22)(q27;q11); CF755: t(3;12)(q27;q11); SO955:der(3)t(3;5)(q27;q31).

Figure 5: Identification of the bcl-6 transcriptional unit. 15 μg of total RNA isolated from the indicated human cell lines was analyzed by Northern blot hybridization using the Sac 4.0 probe (see Figure 2). CB33:EBV-immortalized human B lymphoblastoid cell line; HeLa: human cervical carcinoma cell line; Daudi: human Burkitt lymphoma cell line; Hut78: human T-cell leukemia cell line. Hybridization of the same filter to a mouse GAPDH probe is shown as control for RNA amount loaded in each lane. The faint band comigrating with 28S RNA in all the lanes may be the result of cross-hybridization with ribosomal RNA sequences.

Figure 6: Map of normal human BCL-6 locus. A recombinant genomic DNA library derived from normal placenta DNA was obtained from STRATAGENE Inc and screened by plaque hybridization using the Sac 4.0 probe. Three recombinant phages were obtained (ϕ 1-3 in the figure) whose inserts have been

mapped and shown to overlap on approximately 30 kilobases of genomic DNA representing the BCL-6 locus. These sequences containing bcl-6 exons since they hybridize to the cDNA probe. The precise position of the exons has only been approximately determined and is schematically indicated in the figure. The position of the breakpoints observed in various lymphoma cases is also indicated.

Figure 7: pSac 40 plasmid construction.

Figure 8: pGB31 and pGB3s plasmid construction.

Figures 9A-9D: cDNA and Amino Acid Sequences of BCL-6 (SEQ ID NOs. 1 and 2). The Sac 4.0 probe was used to screen a recombinant phage cDNA library constructed from Bjab B cell lymphoma line RNA. A 4.0 kilobase cDNA was isolated and its nucleotide sequence was determined. It contains a long open reading frame potentially coding for 706 amino acid protein which contains five zinc-finger domains (underlined in the figure; C and H residues which identify the C2H2-type zinc-finger structure are indicated in bold).

Figures 10A-10B:

Structure of BCL-6 cDNA and sequence of its predicted protein product. Figure 10A: Schematic representation of the full-length BCL-6 cDNA clone showing the relative position of the open reading frame (box) with 5′ and 3′ untranslated sequences (lines flanking the box). The approximate positions of the zinc-finger motifs (Zn++) and the NH_2-terminal homology (shaded area) with other proteins are also indicated. Figure 10B: The predicted amino acid

sequence of the BCL-6 protein. The residues corresponding to the six zinc-finger motifs (H-C links). The GenBank Accession number for BCL-6 cDNA and amino acid sequences is U00115.

Figure 11: Homology of the NH_2-terminal region of BCL-6 to other Krüppel zinc-finger proteins, viral (VA55R), or cellular non-zinc-finger (kelch) proteins. Black background indicates identical residues found four or more times at a given position; grey indicates conserved residues that appear in at least four sequences at a given position. Conserved amino acid substitutions are defined according to scheme (P, A, G, S, T), (Q, N, E, D), (H, K, R), (L, I, V, M), and (F< Y< W). Numbering is with respect to the methionine initiation codon of each gene.

Figure 12: Exon-intron organization of the BCL-6 gene and mapping of breakpoints detected in DLCL. Coding and non-coding exons are represented by filled and empty boxes, respectively. The position and size of each exon are approximate and have been determined by the pattern of hybridization of various cDNA probes as well as by the presene of shared restriction sites in the genomic and cDNA. The putative first, second and third exons have been sequenced in the portions overlapping the cloned cDNA sequences. The transcription initiation site has not been mapped (shaded box on 5' side of first exon). Patient codes (e.e. NC11, 891546 etc.) are grouped according to the rearranged patterns displayed by tumor samples. Arrows indicate the breakpoint

position for each sample as determined by restriction enzyme/hybridization analysis. For samples KC1445 and SM1444, the breakpoints have been cloned and the precise positions are known. Restriction sites marked by asterisks have been only partially mapped within the BCL-6 locus. Restriction enzyme symbols are: S, Sac I; B, Bam HI; X, Xba I; H, Hind III, R, Eco RI; G, Bgl II; P, Pst I; Sc, Sca I; St, Stu I; Rs, Rsa I. Tumor samples were collected and analyzed for histopathology at Memorial Sloan-Kettering Cancer Center or at Columbia University.

Figures 13A-13B:

Rearrangements of the BCL-6 gene in diffuse large-cell lymphomas (DLCL). Genomic DNA extracted from tumor biopsies of DLCL cases and from normal lymphocytes (lane N) was digested with the indicated restriction enzymes and analyzed by Southern blot hybridization using the Sac 4.0 probe. Abnormal restriction fragments are indicated by the arrows.

Figures 14A-14C:

Analysis of *BCL-6* rearrangements in AIDS-NHL (Figures 14A-14C). DNAs were digested with *Bam*HI (Figure 14A) or *Xba*I (Figures 14B and 14C) and hybridized to probes Sac4.0 (Figures 14A and 14B) or Sac0.8 (Figure 14C). The BCL-6 germline bands detected by *Bam*HI (11.4 Kb) and *Xba*I (14 Kb) are indicated. U937 was used as a BCL-6 germline control. Among the cases shown, rearrangements were detected in cases DK782, DK827, and DS16, represented by AIDS-DLCL.

Figure 15: Restriction map of the germline *BCL-6* locus. Exon-intron organization of the BCL-6 gene. Coding and noncoding exons are represented by filled and empty boxes, respectively. The transcription initiation site has not been mapped (shaded box on 5′ side of first exon). The breakpoints detected in AIDS-NHL are indicated by arrows. Restriction enzyme symbols are: S, *Sac*I; B, *Bam*HI; X, *Xba*I, R, *Eco*RI. RE, restriction enzyme.

Figures 16A-16C:

Analysis of EBV infection (Figure 16A), c-MYC rearrangements (Figure 16B), and p53 mutations (Figure 16C) in AIDS-NHL. Figure 16A: Analysis of EBV termini heterogeneity in AIDS-NHL. DNAs were digested with *Bam*HI and subjected to Southern hybridization using a DNA probe specific for the fused termini of the EBV genome. U937, a monocytic leukemia cell line, is used as a negative control. A lymphoblastoid cell line derived by EBV infection of normal polyclonal B cells (NC2) is used as control for polymorphic EBV termini. Representative samples of AIDS-NHL, both positive (DK3794, DK4338, DK2814, DK3973) and negative (DK3479), are shown. Figure 16B: Southern blot analysis of c-*MYC* rearrangements in AIDS-NHL. Genomic DNAs from the cases shown was digested with *Hind*III and probed with clone MC413RC[41], representative of c-*MYC* exon 3. A lymphoblastoid cell line (NC2) was used as control for c-*MYC* germline configuration. Among the cases shown, two cases of AIDS-DLCL (DK3537 and DK1446) display a c-*MYC* rearrangement. Figure 16C: Analysis by PCR-SSCP of the p53 gene in

AIDS-NHL. Representative examples are shown for *p53* exon 5. Samples were scored as abnormal when differing from the normal control (N). A sample known to harbor a *p53* mutation was used as positive control (POS). Among the cases shown, DK1171, a case of AIDS-SNCCL, shows a *p53* mutation which was further characterized by direct sequencing of the PCR product.

Figures 17A-17B:

Southern blot analysis of the BCL-6 gene configuration in diffuse large cell lymphomas. Genomic DNA extracted from tumor biopsies was digested with the indicated restriction endonucleases and hybridized using the Sac4.0 probe (19). Rearranged fragments are indicated by the arrows. N = normal control DNA obtained from human lymphocytes.

Figures 18A-18B:

Figure 18A: Freedom from progression in BCL-6 rearranged cases (open circles, top curve) compared to BCL-6 germline cases (closed circles, bottom curve)(P=0.007). Figure 18B: Overall survival from time of diagnosis for BCL-6 rearranged CLLC (open circle, top curve), compared to BCL-6 germline, BCL-2 germline DLLC (dark triangles, middle curve), and BCL-2 rearranged DLLC (dark boxes, bottom curve)(P=0.02).

Figures 19A-19C.

Phosphorylation of BCL-6 by ERK2 *in vitro*: (Fig. 19A) Schematic representation of wild-type and mutant GST-BCL-6 fusion proteins. (*) Serines within MAPK

phosphorylation sites (PXSP). "ZF" zinc finger domain. (Fig. 19B) ERK2 kinase assays using GST-BCL-6 wild-type and deletion mutants as substrates in the presence of [γ-^{32}P]-ATP. (Fig. 19C) (Top) ERK2 kinase assay for wild-type (WT) or mutant (Ala333; Ala333,343) GST-BCL6ΔZF proteins. (Bottom) Commassie Blue staining of the gel shown at top demonstrating comparable amounts of proteins loaded. Molecular weight markers are shown at the left.

Figures 20A-20D.

BCL-6 protein degradation induced by over-expression of MEK-2E in 293T cells. (Fig. 20A) Western blot analysis of 293T cells transfected with 5 μg of BCL-6 (lanes 1, 3) and 5 μg of MEK-2E (lanes 2,3) using anti-BCL-6 (N-70-6; top) or anti-ERK2 (C-14; middle) antibodies. The results of solid phase ERK2 kinase assay performed on cell extract from the same transfectants used in the top. (Fig. 20B) Western blot (top) and Northern blot (bottom) analysis of BCL-6 in 293T cells transfected with pMT2T-BCL-6 (BCL-6) (5 μg) (lanes 1-7) and various amounts of MEK-2E-CMV (MEK-2E) (lanes 2-4) or MEK-CMV (MEK) (5, 10, or 15 μg) (lanes 5-7) as indicated. (Fig. 20C) Western blot analysis of 293T cell extracts transfected with wild-type BCL-6 (lanes 1-3) or BCL-6$_{Ala333,343}$ (lanes 4-6) in the absence (lanes 1, 4) or presence (2, 3, 5, 6) of cotransfected MEK-2E. (Bottom) The results of densitometric scanning of the autoradiography; analogous results were

obtained in three independent experiments. (Fig. 20D) Analysis of BCL-6 transrepression activity in the presence of active MEK. 293T cells were transfected with 2.0 pmol of B6BS-TK-Luc, 0.04 pmol of pMT2T-BCL-6 (lanes 2-8) or pMT2T-BCL-$6_{Ala333,343}$ (lanes 11-14) and increasing amounts (0.1, 0.2, 0.4, 0.4 pmol) of MEK-2E (lanes 3-5, 9, 12-14) or MEK (lanes 6-8, 10) as indicated. Cells were harvested 48 hrs after transfection and luciferase activities were measured by a luminometer.

Figures 21A-21B.

BCL-6 contains PEST sequences which are required for phosphorylation-induced degradation. (Fig. 21A). Schematic representation of HA-tagged BCL-6 proteins. PEST sequences were identified by the PEST-FIND program. PEST1: AA336-AA351 (KSDCQPNSPTESCSSK) (SEQ ID NO:10), score 9.4; PEST2: AA365-AA371(KSPTDPK) (SEQ ID NO:11), score 5.0; PEST3: AA406-AA430 (RAYTAPPACQPPMEPENLDLQSPTK) (SEQ ID NO:12), score 2.6. (Fig. 21B) 293T cells were transfected with 5 μg of pMT2T vectors expressing HA-BCL-6 (lanes 1-3), HA-BCL-6Δ(300-417) (lanes 4-6), or HA-BCL-6ZF (lanes 7-9) in the absence of MEK-2E (lanes 1, 4, 7) or in the presence of increasing amount (5, 10 μg) of MEK-2E (lanes 2, 5, 8, 3, 6, 9). Forty-eight hrs after transfection, equal amounts of cell lysates were analyzed (after normalization for transfection efficency based on β-galactosidase activity of co-transfected

plasmids) by 8% SDS-PAGE and Western blot using anti-HA (12CA5) antibodies.

Figures 22A-22B

MAPK-induced BCL-6 degradation is mediated by the ubiquitin/proteasome pathway. (Fig. 22A) Western blot analysis of BCL-6 proteins in 293T cells transfected with BCL-6 in the absence or presence of co-transfected MEK-2E treated with 0.2% DMSO (lanes 1-3), 50 μM Calpain inhibitor II (lanes 4-6), or 50 μM MG132 (lanes 7-9) (added 8hrs after transfection). (Fig. 22B) 293T cells were transfected with BCL-6, His_6-Ub, and MEK-2E in the absence (lanes 1-4) or presence of MG132 (lanes 5-8). Cell lysates were immunoprecipitated with anti-BCL-6 antibodies (N-70-6) and the immunoprecipitants were analyzed by 6% SDS-PAGE followed by Western blot analysis using anti-ubiquitin antibodies.

Figures 23A-23E.

BCL-6 is phosphorylated and degraded by antigen receptor signaling in B cells. Ramos cells ($1x10^6$/ml) were treated with anti-IgM (10 g/ml) and harvested at different time points after treatment as indicated. (Fig. 23A) (Top three panels) Equal amounts of cell extracts were used for Western blot analysis using anti-BCL-6 (top), or anti-ERK2 (middle) antibodies, and for solid phase ERK2 kinase assays (MBP, bottom); Equal amounts of RNAs (10 μg) were used for Northern blot analysis with BCL-6 or GAPDH probes (bottom). (Fig. 23B) Hyperphosphorylated BCL-6 proteins are

more unstable. Ramos cells were pulse labeled for 1 hour with [^{35}S]methionine and [^{35}S]cysteine, and then treated with anti-IgM (10 μg/ml) for 30 min and subsequently incubated in the presence of an excess of nonradioactive methionine and cysteine for the indicated times (chase). Cell extracts were immunoprecipitated with anti-BCL-6 antibodies and analyzed by SDS-PAGE followed by autoradiography. (Fig. 23C) Anti-IgM induced BCL-6 phosphorylation and degradation is prevented by a specific MEK inhibitor. Western blot analysis of BCL-6 in Ramos cells treated with anti-IgM in the presence of 0.2% DMSO or 50 μM PD098059 (added 30 min before anti-IgM treatment). (Fig. 23D) Anti-IgM induced BCL-6 degradation is prevented by a specific proteasome inhibitor. Western blot analysis of BCL-6 in Ramos cells treated with anti-IgM in the presence of 0.2% DMSO (lanes 2-4), 50 μM Calpain inhibitor II (lanes 5-7), and 50 μM MG132 (lanes 8-10) (added 1 hr before the treatment). (Fig. 23E) Mutant BCL-6 proteins are resistant to anti-IgM-induced degradation. Ramos cells stably transfected with pHeBo-MT-HA-BCL6, pHeBo-MT-HA-BCL-6$_{Ala333,343}$ and pHeBo-MT-HA-BCL6ZF were treated with 1 μM $CdCl_2$ for 6 hrs to induce exogenous BCL-6 expression. Cells were then treated with anti-IgM (10 μg/ml) and harvested at different time points as indicated. Equal amounts of cell extracts were loaded on 7% (HA-BCL-6 or HA-BCL-6$_{Ala333,343}$) or 10% SDS-PAGE (HA-BCL-6ZF) and the amount of exogenous BCL-6 proteins were analyzed by

Western blot using anti-HA antibodies (12CA5).

DETAILED DESCRIPTION OF THE INVENTION

The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

C=cytosine	A=adenosine
T=thymidine	G=guanosine

This invention provides an isolated vertebrate nucleic acid molecule of the bcl-6 locus. As used herein, bcl-6 locus means the breakpoint cluster region in B-cell lymphomas. The bcl-6 locus is of 30 kilobase in length containing at least a bcl-6 gene which codes for a protein. Therefore, the bcl-6 locus contains both the 5′ and 3′ flanking region of the coding sequences of the bcl-6 gene.

In an embodiment, the isolated, vertebrate nucleic acid molecule of bcl-6 locus is DNA. In another embodiment, the isolated, vertebrate nucleic acid of the bcl-6 locus is cDNA. In a further embodiment, the isolated, vertebrate nucleic acid is genomic DNA. In a still further embodiment, the isolated, vertebrate nucleic acid molecule is RNA.

This invention provides an isolated, human nucleic acid molecule comprising the bcl-6 locus.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

Moreover, the isolated vertebrate nucleic acid molecules

are useful for the development of probes to study B cell lymphomas.

This invention provides a nucleic acid molecule comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of the bcl-6 locus. In an embodiment, this molecule is DNA. In another embodiment, the molecule is RNA.

As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs.

The above nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of bcl-6 locus may be used as a probe for bcl-6 sequences. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule having the full-length or a fragment of the bcl-6 locus into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

RNA probes may be generated by inserting the full length or a fragment of the bcl-6 locus downstream of a bacteriophage promoter such as T3, T7 or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with a linearized bcl-6 or its fragment where it contains an

upstream promoter in the presence of the appropriate RNA polymerase.

This invention provides an cDNA molecule of bcl-6 locus operatively linked to a promoter of RNA transcription.

This invention provides a vector which comprises the nucleic acid molecule of bcl-6 locus. This invention provides the above vector, wherein the isolated nucleic acid molecule is linked to a plasmid.

This invention further provides isolated cDNA molecule of the bcl-6 locus operatively linked to a promoter of RNA transcription. Various vectors including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses are well known to ordinary skilled practitioners.

As an example to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules which base pair with each other and are then ligated together with DNA ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other means are also available and known to an ordinary skilled practitioner.

In an embodiment, a partial cDNA molecule of the bcl-6 locus is linked to pGEM-7zf(-) and the resulting plasmid is designated as pGB31 (Figure 8). Plasmid, pGB31 was deposited on June 3, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, pGB31 was accorded with ATCC Accession Number 75476.

In an another embodiment, a partial cDNA molecule of the bcl-6 locus is linked to pGEM-7zf(-) and the resulting plasmid is designated as pGB3s (Figure 8). Plasmid, pGB3s was deposited on June 3, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, pGB3s was accorded with ATCC Accession Number 75477.

This invention provides a host vector system for the production of a polypeptide encoded by bcl-6 locus, which comprises the above vector in a suitable host.

This invention provides the above host vector system, wherein the suitable host is a bacterial cell, insect cell, or animal cell.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well-known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the polypeptide encoded by the bcl-6 locus.

This invention further provides an isolated DNA or cDNA molecule described hereinabove wherein the host cell is

selected from the group consisting of bacterial cells (such as E.coli), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

This invention provides a method of producing a polypeptide encoded by bcl-6 locus, which comprises growing the above host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

This invention provides a polypeptide encoded by the isolated vertebrate nucleic acid molecule of bcl-6 locus.

This invention provides an antibody capable of binding to polypeptide encoded by bcl-6 locus. In an embodiment, the antibody is monoclonal.

This invention provides a method to select specific regions on the polypeptide encoded by the bcl-6 locus to generate antibodies. The protein sequence may be determined from the cDNA sequence. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer of the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment. Usually, the hydrophilic regions will be more immunogenic than the hydrophobic regions. Therefore the hydrophilic amino acid sequences may be selected and used to generate antibodies specific to polypeptide encoded by the bcl-6 locus. The selected peptides may be prepared using commercially available machines. As an alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and

used as an immunogen.

Polyclonal antibodies against these peptides may be produced by immunizing animals using the selected peptides. Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Alternatively, monoclonal antibodies may be produced by in vitro techniques known to a person of ordinary skill in the art. These antibodies are useful to detect the expression of polypeptide encoded by the bcl-6 locus in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

The antibody may be labelled with a detectable marker, including but not limited to: a radioactive label, or a calorimetric, luminescent, or fluorescent marker, or gold. Radioactive labels include but are not limited to: ^{3}H, ^{14}C, ^{32}P, ^{33}P; ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{59}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re. Fluorescent markers include but are not limited to: fluorescein, rhodamine and auramine. Methods of producing the polyclonal or monoclonal antibody are known to one of ordinary skill in the art.
Further, the antibody complex may be detected by a second antibody which may be linked to an enzyme, such as alkaline phosphatase or horseradish peroxidase. Other enzymes which may be employed are well known to one of ordinary skill in the art.

This invention provides for the isolated nucleic acid molecule of bcl-6 that is labelled with a detectable marker. The detectable marker may be a radioactive label, a calorimetric, luminescent, or a fluorescent marker. Other detectable markers are known to those skilled in the art as hereinabove described.

This invention provides an antagonist capable of blocking

the expression of the polypeptide encoded by the isolated nucleic acid molecule of bcl-6. The antagonist may be a triplex oligonucleotide capable of hybridizing to nucleic acid molecule bcl-6.

This invention provides an antisense molecule capable of hybridizing to the nucleic acid molecule bcl-6. The antisense molecule may be DNA or RNA.

This invention provides a triplex oligonucleotide capable of hybridizing with a double stranded DNA molecule bcl-6.

The antisense molecule may be DNA or RNA or variants thereof (i.e. DNA with a protein backbone). The present invention extends to the preparation of antisense nucleotides and ribozymes that may be used to interfere with the expression of the receptor recognition proteins at the translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or cleaving it with a ribozyme.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule. In the cell, they hybridize to that mRNA, forming a double stranded molecule. The cell does not translate an mRNA in this double-stranded form. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Oligomers of about fifteen nucleotides and molecules that hybridize to the AUG initiation codon will be particularly efficient, since they are easy to synthesize and are likely to pose fewer problems than larger molecules upon introduction to cells.

This invention provides a transgenic nonhuman mammal which comprises the isolated nucleic acid molecule bcl-6 introduced into the mammal at an embryonic stage.

This invention provides an assay for non-Hodgkin's

lymphoma, comprising (a) incubating a sample of suitable body fluid for a subject with a monoclonal antibody reactive with non-Hodgkin's lymphoma cells to a solid support, (b) removing unbound body fluid from the support, and (c) determining the level of antigen activity exhibited by the bound body fluid to the support.

The suitable bodily fluid sample is any bodily fluid sample which would contain non-hodgkin lymphoma cells or fragments thereof. A suitable bodily fluid includes, but is not limited to, serum, plasma, cerebrospinal fluid, and urine. In the preferred embodiment, the suitable bodily fluid sample is serum or plasma. In addition, the body fluid sample may cells from bone marrow, or a supernate from a cell culture. Methods of obtaining a suitable bodily fluid sample from a subject are known to those skilled in the art.

This invention provides a method for screening putative therapeutic agents for treatment of non-Hodgkin's lymphoma, which comprises determining in a first sample from a subject with non-Hodgkin's lymphoma the presence of the isolated nucleic acid molecule bcl-6, administering to the subject a therapeutic amount of the agent such that the agent is contacted with the cell associated with the condition, determining after a suitable period the amount of the isolated nucleic acid molecule in a sample from the treated subject, and comparing the amount of isolated nucleic acid molecule determined in the first sample with the amount determined in the sample from the treated subject, a difference indicating the effectiveness of the agent, thereby screening putative therapeutic agents for treatment of non-Hodgkin's lymphoma.

Further, this invention provides an assay system that is employed to identify drugs or other molecules capable of binding to the nucleic acid molecule bcl-6 or proteins, either in the cytoplasm or in the nucleus, thereby

inhibiting or potentiating transcriptional activity. Such assay would be useful in the development of drugs that would be specific against particular cellular activity, or that would potentiate such activity, in time or in level of activity.

The above described probes are also useful for in-situ hybridization or in order to locate tissues which express this gene, or for other hybridization assays for the presence of this gene or its mRNA in various biological tissues.

The in-situ hybridization technique using the labelled nucleic acid molecule bcl-6 is well known in the art. Essentially, tissue sections are incubated with the labelled nucleic acid molecule to allow the hybridization to occur. The molecule will carry a marker for the detection because it is "labelled", the amount of the hybrid will be determined based on the detection of the amount of the marker. Further, immunohistochemical protocols may be employed which are known to those skilled in the art.

This invention provides a method of diagnosing diffuse-type B-cell lymphoma in a subject which comprises detecting in a sample from the subject nucleic acid molecule of bcl-6 locus.

This invention provides a method for diagnosing B-cell lymphoma in a subject comprising: (a) obtaining DNA sample from the subject; (b) cleave the DNA sample into fragments; (c) separating the DNA fragments by size fractionation; (d) hybridizing the DNA fragments with a nucleic acid molecule comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of the nucleic acid molecule of the bcl-6 locus to detect the DNA fragment containing the bcl-6 sequence; and (e) comparing the

detected DNA fragment from step (d) with the DNA fragment from a known normal subject, the difference in size of the fragments indicating the occurrence of B-cell lymphoma in the subject. In a preferred embodiment, the above diagnostic method is for diffuse-type B-cell lymphomas.

A person of ordinary skill in the art will be able to obtain appropriate DNA sample for diagnosing B-cell lymphoma in a subject. The DNA sample obtained by the above described method may be cleaved by restriction enzyme. The uses of restriction enzymes to cleave DNA and the conditions to perform such cleavage are well-known in the art.

In an embodiment, the size fractionation in step (c) of the above-described method is effected by a polyacrylamide gel. In another embodiment, the size fractionation is effected by an agarose gel.

This invention also provides the above-described diagnosis method wherein step the nucleic acid molecule in step (d) is labeled with a detectable marker. The detectable marker includes but is not limited to a radiolabelled molecule, a fluorescent molecule, an enzyme, or a ligand.

In a preferred embodiment, the above-described diagnosis method further comprises transferring the DNA fragments into a solid matrix before the hybridization step (d). One example of such solid matrix is nitrocellulose paper.

As an example for the above-described diagnosis method is shown in Figures 4A-4C where different NHL sample are analyzed. More lymphoma cases and their breakpoints are shown in Figure 6.

This invention also provides a method for diagnosing B-cell lymphoma in a subject comprising: (a) obtaining RNA sample from the subject; (b) separating the RNA sample into

different species by size fractionation; (c) hybridizing the RNA species with a nucleic acid molecule comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of the nucleic acid molecule of the bcl-6 locus to detect the RNA species containing the bcl-6 sequence; and (d) comparing the RNA species obtained from (c) with the RNA species from a known normal subject, the difference in size of the species indicating the occurrence of B-cell lymphoma in the subject.

In an embodiment, the size fractionation in step (b) is effected by a polyacrylamide or agarose gel.

This invention also provides the above-described method where in step (c), the nucleic acid molecule is labeled with a detectable marker. The detectable marker includes but is not limited to a radiolabelled molecule, a fluorescent molecule, an enzyme, or a ligand.

This invention also provides the above-method further comprises transferring the RNA species into a solid matrix before step (c).

This invention also provides various uses of bcl-6 locus/gene an its derivatives. This invention further provides a method for diagnosis of B cell lymphoma and/or diffuse-type B cell lymphoma using bcl-6 DNA probes or synthetic oligonucleotide primers derived from bcl-6 sequences to detect bcl-6 rearrangements/mutations by Southern blotting PCR or other DNA based techniques.

This invention also provides a method of diagnosis of B cell lymphoma and/or diffuse-type B cell lymphoma using bcl-6 DNA probes or synthetic oligonucleotide primers derived from bcl-6 sequences to detect abnormal bcl-6 RNA species by Northern blotting, PCR or other RNA-based techniques.

This invention further provides a method of diagnosis of B cell lymphoma and/or diffuse-type B cell lymphoma using antiserum or monoclonal antibodies directed against the bcl-6 protein product(s).

This invention provides a method of treating a subject with non-Hodgkin's lymphoma comprising administering an effective amount of the antisense molecule of the nucleic acid molecule bcl-6 operatively linked to a suitable regulatory element coupled with a therapeutic DNA into a tumor cell of a subject, thereby treating the subject with non-Hodgkin's lymphoma.

This invention provides a method of treating a subject with non-Hodgkin's lymphoma, comprising administering an effective amount of the antagonist capable of blocking the expression of the polypeptide encoded by the isolated nucleic acid molecule of bcl-6, and a suitable acceptable carrier, thereby treating the subject with non-Hodgkin's lymphoma.

Further, as is known to those of ordinary skill in the art effective amounts vary with the type of therapeutic agent. It is known to those of ordinary skill in the art how to determine an effective amount of a suitable therapeutic agent.

The preparation of therapeutic compositions which contain polypeptides, analogs or active fragments as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose,

glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

A polypeptide, analog or active fragment can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The subjects contained herein may be a mammal, or more specifically a human, horse, pig, rabbit, dog, monkey, or rodent. In the preferred embodiment the subject is a human.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount: Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual.

Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration.

As used herein administration means a method of

administering to a subject. Such methods are well known to those skilled in the art and include, but are not limited to, administration topically, parenterally, orally, intravenously, intramuscularly, subcutaneously or by aerosol. Administration of the agent may be effected continuously or intermittently such that the therapeutic agent in the patient is effective to treat a subject with non-hodgkin's lymphoma.

Finally, this invention provides a therapy of B cell lymphoma and/or diffuse-type B cell lymphoma using anti bcl-6 reagents including specific antisense sequences and compounds interfering with bcl-6 functions.

This invention further provides a method of degrading BCL-6 in cells comprising administering a molecule which induces phosphorylation of BCL-6 and thereby induces BCL-6 degradation. In an embodiment of the above-described method of the molecule which induces phosphorylation of the BCL-6 is a mitogen-activated protein kinase (MAPK). In another embodiment of the method of the molecule which induces phosphorylation of the BCL-6 is a functionally active mutant of a mitogen-activated protein kinase (MAPK). In an embodiment of the method the MAPK includes but is not limited to ERK-1 or ERK-2. In an embodiment the BCL-6 is phosphorylated either at one phosphorylation site or at multiple sites. In a preferred embodiment of the method, the molecule which induces phosphorylation of the BCL-6 is a molecule which activates an antigen receptor on B cell surfaces. In an embodiment the molecule which activates an antigen receptor on B cell surfaces is an antibody. In an embodiment the antibody includes but is not limited to an anti-IgM antibody. The antibody may also be an anti-idiotypic antibody which activates an antigen receptor on B cell surfaces. In another embodiment of the above-described method the molecule which activates an antigen receptor on B cell surfaces is a molecule which activates MAPK in B cells. As used herein "activation of an antigen

receptor on B cell surfaces" is defined as induction of signal transduction including MAPK activation. In a preferred embodiment of the method the molecule which activates MAPK in B cells is a cytokine. In a further preferred embodiment of the method the cytokine used includes but is not limited to TNF, IL-6, or IL-2. In an embodiment of the method the molecule is cross-linked to a B cell antigen receptor to activate the receptor. In an embodiment cross-linking the molecule to the B cell antigen receptor activates the MAPK.

This invention provides a method of treating a subject with lymphoma which comprises administering an effective amount of a pharmaceutical composition comprising a molecule which induces phosphorylation of BCL-6 protein so as to induce degradation of BCL-6 and a pharmaceutically acceptable carrier, thereby treating the subject with lymphoma. In an embodiment of the above-described method the lymphoma expresses BCL-6. In a preferred embodiment of the method the pharmaceutical composition comprises a MAPK activator. In a further preferred embodiment the MAPK activator is an antibody. In another embodiment of the method the antibody used includes but is not limited to an anti-IgM antibody. The antibody may also be an anti-idiotypic antibody which activates an antigen receptor on B cell surfaces. In another embodiment the MAPK activator is a cytokine. The cytokine used may be selected from but is not limited to an cytokines such as TNF, IL-6, or IL-2. In a preferred embodiment the lymphoma is a B-cell lymphoma. In another preferred embodiment the B-cell lymphoma is derived from germinal center B cells. In an embodiment of the above-described method the administration of the pharmaceutical composition may be selected from but is not limited to intravenous or intratumor administration.

This invention provides a method of deceasing BCL-6 levels in cells comprising administering a compound which interferes with transcription of bcl-6 and thereby prevents

expression of BCL-6 protein so as to thereby deceasing BCL-6 levels in the cells. In an embodiment of the above-described method the compound which interferes with transcription of bcl-6 prevents binding of a transcription factor and histone acetylase/deacetylase complexes.

In another embodiment the compound is N,N'-hexamethylene bisacetamide (HMBA) or trichostatin. Applicants incorporate by reference U.S. Patent 5,608,108 issued March 4, 1997 (Marks et al.), U.S. Patent 5,175,191 issued December 29, 1992 (Marks et al.), and U.S. Patent 5,055,608 issued October 8, 1991 (Marks et al.) for compounds disclosed therein which interfere with transcription. In an embodiment the method of treating lymphoma comprising decreasing BCL-6 levels in cells comprises the above-described method of deceasing BCL-6 levels in cells comprising administering a compound which interferes with transcription of bcl-6 and thereby prevents expression of BCL-6 protein so as to thereby deceasing BCL-6 levels in the cells.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAIL SECTION I:

Materials and Methods

DNA Extraction and Southern Blot Analysis. Total genomic DNA was purified from frozen tumor biopsies by cell lysis, proteinase K digestion, "salting-out" purification and ethanol precipitation as previously described (11). Southern blot hybridization analysis was performed in 50% formamide, 3X SSC, 10X dextran sulphate, 5X Denhardt's solution, 0.5% SDS at 37°C for 16 hrs. Filters were washed in 0.2X SSC, 0.5% SDS at 60°C for 2 hrs. DNA probes were ^{32}P-labelled by the random priming method (12).

DNA Probes. The following probes were used for Southern blot analysis of Ig gene rearrangements: i) (J_H) probe: 6.6 kb *Bam*Hi/*Hind*III fragment from the human Ig heavy-chain (Ig_H) locus (13); ii) (C_μ) probe: 1.3 kb *Eco*RI fragment containing the first two exons of human C_μ (13).

Genomic Cloning. Genomic libraries from NHL cases SM1444 and KC1445 were constructed by partial Sau 3A restriction digestion of genomic DNA and ligation of gel-purified 15-20 kb fractions into LambdaGem-11 phage vector (Promega). Library screening was performed by plaque-hybridization using the C_μ probe.

Fluorescence in situ Hybridization Analysis (FISH). Phage DNA was labelled with biotin-14-dATP by nick translation and hybridized to metaphase spreads from normal human lymphocytes as described (14). To visualize the hybridization signal and the corresponding bands sequentially under the microscope, the slides were stained and counterstained with propidium iodide and 4'6'-diamideno-2-phenylindole (DAPI), respectively.

Northern Blot Hybridization Analysis. RNAs from several human cell lines were extracted by the guanidine-

isothiocyanate method (15). For Northern blot analysis, RNA samples were electrophoresed through 0.9% agarose-2.2M formaldehyde gels and then transferred to nitrocellulose filters. Hybridization and washing were performed as described for Southern blot analysis.

Experimental Results:

DNA was extracted from tumor tissue of two cases (SM1444 and KC1445) of IgM-producing, diffuse-type B-cell NHL carrying the t(3;14)(q27;q32) translocation. Since the involvement of the Ig_H locus was suspected based on the 14q32 breakpoint, SM1444 and KC1445 DNAs were first analyzed by Southern blot hybridization using combinations of enzymes and probes specific for the J_H and C_μ regions of the Ig_H locus (13). In both cases, digestion by *Bam*HI showed rearranged fragments containing J_H sequences (Figure 1). Subsequent hybridizations to the C_μ probe showed, in each case, that one rearranged fragment containing J_H sequences was not linked to C_μ sequences (see failure of the C_μ probe to hybridize to the same rearranged *Bam*HI fragment detected by J_H (Figure 1) as would be expected for the physiologically rearranged Ig_H allele in IgM producing cells. In addition, in both cases, digestion with *Hind*III and hybridization with C_μ detected a rearranged fragment, a finding inconsistent with either germ-line or physiologically rearranged Ig_H genes, since both *Hind*III sites flanking C_μ sequences are not involved in V-D-J arrangements (13). The observed pattern is, however, consistent with chromosomal breakpoints located within C_μ switch sequences, as previously observed in several cases of chromosomal translocations involving the Ig_H locus (2,16-18).

Based on this analysis, the C_μ containing fragments from each case were cloned by screening genomic libraries constructed from SM1444 and KC1445 DNAs using the C_μ probe. Restriction mapping and hybridization analysis of several

phage clones led to the identification of recombinant phages from each library which contained C_μ sequences linked to sequences unrelated to the Ig_H locus (see Figure 2 for maps of representative phage clones). The Ig portions of the phage inserts overlapped along the C_μ region extending 5' into the switch region where alignment with the restriction map of the normal Ig heavy-chain locus was lost. The location of the breakpoint within C_μ switch sequences was confirmed for case SM1444 by DNA sequence analysis of the breakpoint junction of phage SM-71, which revealed the presence of the repeated motifs typical of the Ig_H switch regions on the chromosome 14 side (19). The Ig-unrelated portions of phage SM-71 and KC-51 also overlapped with each other in their restriction maps, suggesting that they were derived from the same genomic region. This notion is further supported by the fact that probe Sac 4.0 derived from SM-71 was able to hybridize to the corresponding region of KC-51 in Southern blot analysis.

To determine the chromosomal origin of the Ig-unrelated sequences, a recombinant phage (SM-71) derived from case SM1444, was used as a probe in FISH analysis on metaphase chromosome spreads from mitogen-stimulated normal blood lymphocytes. The phage probe hybridized specifically to chromosome 14q32 as well as to chromosome 3q27 (Figure 3), indicating that the recombinant phage insert contained one of the two chromosomal junctions of the reciprocal t(3;14) translocation. Thus, taken together, the results of cloning and FISH analysis established that, in both NHL cases studied, the chromosomal translocation has linked sequences within the switch region of the C_μ locus to sequences from band 3q27, consistent with the cytogenetic description of the t(3;14)(q27;q32) translocation. In the two NHL cases studied, the breakpoints on 3q27 were located within 3 kb of the same genomic locus, which was termed <u>bcl-6</u>.

In order to determine whether 3q27 breakpoints in

additional NHL cases were also located within the cloned portion of the bcl-6 locus, bcl-6 rearrangements were examined in a total of 19 NHL cases carrying 3q27 breakpoints, including 4 (two cloned cases and two additional ones) carrying t(3;14)(q27;q32) as well as 15 cases carrying 3q27 translocations involving regions other than 14q32. Southern blot hybridization using probes derived from phage SM-71 (see Figure 2) detected rearranged fragments in *Eco*RI-and/or *Bgl*II-digested DNA in 7 of 19 cases studied, including all 4 t(3;14) cases as well as 3 cases with other types of translocations (see Figures 4A-4C for cytogenetic description of the cases and representative results). These results indicate that heterogeneous 3q27 breakpoints cluster in a fairly restricted region within bcl-6 independently of the partner chromosome involved in the translocation.

Whether the bcl-6 locus adjacent to the chromosomal breakpoints contained a transcriptional unit was investigated. Probe Sac 4.0 (see Figure 2) was used to detect RNA expression in several human cell lines by Northern blot analysis. A major 2.4 kb RNA species was readily detectable in two B-cell derived cell lines tested, while a relatively less abundant 4.4 kb species is present in CB33 only. No hybridization was detected in a T-cell derived cell line (HUT 78) nor in HeLa cells (Figure 5). This result indicates that 3q27 sequences immediately adjacent to the chromosomal breakpoint cluster are part of a gene (bcl-6) which is expressed in cells of the B lineage.

Experimental Discussion:

This study reports the identification and cloning of a genomic region, bcl-6, involved in recurrent chromosomal translocations affecting band 3q27 in NHL. The region is defined by the clustered position of breakpoints in seven NHL cases carrying 3q27 translocations involving either IgH

or several other loci. A more precise definition of the bcl-6 locus and of the frequency of its involvement in NHL requires cloning and characterization of additional bcl-6 sequences and studying additional tumor cases. Nevertheless, the finding that various translocation partner chromosomes have been joined to the same region on chromosome 3 in cytogenetically heterogenous NHL cases supports the notion that rearrangement of the bcl-6 locus may represent the critical common denominator of translocations involving 3q27.

The second finding of this study is that the bcl-6 locus contains a gene which is expressed in B-cells. It is not clear at this stage whether the chromosomal breakpoints directly truncate coding or regulatory sequences of bcl-6, or, whether the gene remains intact with its regulation overridden by transcriptional control motifs juxtaposed by the translocation. The clustering of breakpoints in the seven studied NHL cases suggests, however, that bcl-6 may be a proto-oncogene which can contribute to NHL pathogenesis upon activation by chromosomal translocation. Results of this study will allow elucidation of the normal structure and function of the bcl-6 gene in order to understand the pathogen consequences of chromosomal translocation of bcl-6 and its role in lymphomagenesis.

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EXPERIMENTAL DETAIL SECTION II

Introduction:

The molecular analysis of specific chromosomal translocations has improved the understanding of the pathogenesis of non-Hodgkin lymphoma (NHL), a heterogeneous group of B-cell or, less frequently, T-cell malignancies (1,2). The (14;18) chromosomal translocation, which causes the deregulated expression of the anti-apoptosis gene BCL-2, plays a critical role in the development of follicular lymphoma (FL) (3-6), which accounts for 20 to 30% of all NHL diagnoses (7). Burkitt's lymphoma (BL) and mantle-cell lymphoma, two relatively rare NHL types, are characterized by chromosomal translocations causing the deregulated expression of the cell-cycle progression genes C-MYC and the BCL-1/cyclin D1, respectively (8-15).

Relatively little is known about the molecular pathogenesis of diffuse large cell lymphoma (DLCL), the most frequent and most lethal human lymphoma (7). DLCL accounts for ~40% of initial NHL diagnoses and is often the final stage of progression of FL(7). A small percentage of DLCL display C-MYC rearrangements (16) and 20 to 30% display alterations of BCL-2 reflecting the tumor's derivation from FL (17). However, no consistent molecular alteration has been identified that is specific for DLCL.

Chromosomal translocations involving reciprocal recombinations between band 3q27 and several other chromosomal sites are found in 8 to 12% of NHL cases, particularly in DLCL (18-19). From NHL samples displaying recombinations between 3q27 and the immunoglobulin (Ig) heavy chain locus on 14q32, the chromosomal junctions of several (3;14)(q27;q32) translocations were cloned and identified a cluster of breakpoints at a 3q27 locus named BCL-6.

Experimental Results:

To isolate normal BCL-6 cDNA, a cDNA library constructed form the NHL cell line Bjab (22) was screened with a probe (20-21) derived from the chromosomal region flanking the breakpoints of two t(3;14)(q27;32) cases. A phage cDNA library constructed from RNA of the Bjab lymphoma cell line was screened (1 X 10^6 plaques) by plaque hybridization with the Sac 4.0 probe that had been ^{32}P-labelled by random priming (22). Sequence analysis (Figures 10A-10B) revealed that the longest clone (3549 bp), approximately the same size as BCL-6 RNA, codes for a protein of 706 amino acids with a predicted molecular mass of 79kD. The putative ATG initiation codon at position 328 is surrounded by a Kozak consensus sequence (23) and is preceded by three upstream in-frame stop codons. The 1101-bp 3'-untranslated region contains a polyadenylation signal followed by a track of poly(A). These features are consistent with BCL-6 being a functional gene.

The NH_2- and COOH- termini of the BCL-6 protein (Figures 10A-10B) have homologies with "zinc-finger" transcription factors (24). BCL-6 contains six C_2H_2 zinc-finger motifs (Figure 10A) and a conserved stretch of six amino acids (the H/C link) connecting the successive zinc-finger repeats (25), BCL-6 can be assigned to the Krüppel-like subfamily of zinc-finger proteins. The NH_2- terminal region of BCL-6 is devoid of the FAX (27) and KRAB (28) domains sometimes seen in Krüppel-related zinc-finger proteins, but it does have homologies (Figure 11) with other zinc-finger transcription factors including the human ZFPJS protein, a putative human transcription factor that regulates the major histocompatibility complex II promoter, the Tramtrack (ttk) and Broad-complex (Br-c) proteins in Drosophila that regulate developmental transcription (29), the human KUP protein (31), and the human PLZF protein, which is occasionally involved in chromosomal translocations in human promyelocytic leukemia (32). The regions of NH_2-

terminal homology among ZFPJS, ttk, Br-c, PLZF and BCL-6 also share some degree of homology with viral proteins (e.g. VA55R) of the poxvirus family (33) as well as with the Drosophila kelch protein involved in nurse cell-oocyte interaction (34). These structural homologies suggest that BCL-6 may function as a DNA-binding transcription factor that regulates organ development and tissue differentiation.

The cDNA clone was used as a probe to investigate BCL-6 RNA expression in a variety of human cell lines by Northern blot analysis. A single 3.8 kb RNA species was readily detected (Figure 11) in cell lines derived from mature B-cells, but not from pro-B-cells or plasma cells, T cells or other hematopoietic cell lineages. The BCL-6 RNA was not detectable in other normal other tissues, except for skeletal muscle in which low level expression was seen. Thus, the expression of BCL-6 was detected in B-cells at a differentiation stage corresponding to that of DLCL cells. This selective expression in a "window' of B-cell differentiation suggests that BCL-6 plays a role in the control of normal B-cell differentiation and lymphoid organ development.

To characterize the BCL-6 genomic locus, the same cDNA probe to screen a genomic library from human placenta was used. A phage genomic library constructed from normal human placenta DNA (Stratagene) was screened (8×10^5 plaques) with the BCL-6 cDNA. Twelve overlapping clones spanning ~50kb of genomic DNA were isolated. After restriction mapping, the position of various BCL-6 exons was determined by Southern hybridization using various cDNA probes. By restriction mapping, hybridization with various cDNA probes, and limited nucleotide sequencing, the BCL-6 gene was found to contain at least ten exons spanning ~26 kb of DNA (Figure 12). Sequence analysis of the first and second exons indicated that they are noncoding and that the translation initiation codon is within the third exon.

Various cDNA and genomic probes were used in Southern (DNA) blot hybridizations to determine the relationship between 3q27 (Table 1). Monoallelic rearrangements of BCL-6 were detected in 12 of 17 tumors by using combinations of restriction enzymes (Bam HI and Xba I) and probe which explore ~16 kb within the BCL-6 locus. These 12 positive cases carry recombinations between 3q27 and several different chromosomes (Table 1), indicating that heterogeneous 3q27 breakpoints cluster in a restricted genomic locus irrespective of the partner chromosome involved in the translocation. Some DLCL samples (5 of 17) do not display BCL-6 rearrangements despite cytogenetic alterations in band 3q27, suggesting that another gene is involved or, more likely, that there are other breakpoint clusters 5′ or 3′ to BCL-6. If the latter is true, the observed frequency of BCL-6 involvement in DLCL (33%, see below) may be an underestimate.

Table 1.

Frequency of BCL-6 rearrangements in DLCL carrying chromosomal translocations affecting band 3q27

Translocation	Fraction of tumors with BCL-6 rearrangements
t(3;14(q27;q32)	4/4
t(3;22)(q27;q11)	2/3
t(3;12)(q27;q11)	1/1
t(3;11)(q27;q13)	1/1
t(3;9)(q27;p13)	0/1
t(3;12)(q27;q24)	0/1
der(3)t(3;5)(q27;q31)	1/1
t(1;3)(q21;q27)	1/1
t(2;3)(q23;q27)	1/1
der(3)t(3;?)(q27;?)	1/3

Tumor samples listed in the Table were collected and analyzed for histopathology and cytogenetics at Memorial Sloan-Kettering Cancer Center.

A panel of tumors not previously selected on the basis of 3q27 breakpoints but representative of the major subtypes of NHL as well as of other lymphoproliferative diseases was analyzed. Similar rearrangements were detected in 13 of 39 DLCL, but not in other cases including other NHL subtypes (28 FL, 20 BL, and 8 small lymphocytic NHL), acute lymphoblastic leukemia (ALL; 21 cases), and chronic lymphocytic leukemia (CLL; 31). These findings indicate that BCL-6 rearrangements are specific for and frequent in DLCL. In addition, the frequency of rearrangements in DLCL (33%) significantly exceeds that (8 to 12%) reported at the cytogenetic level, suggesting that some of the observed rearrangements may involve submicroscopic chromosomal alterations undetectable at the cytogenetic level.

All the breakpoints in BCL-6 mapped to the putative 5' flanking region, the first exon or the first intron (Figure 12). For two patients that carry (3;12)(q27;q32) translocations, the chromosomal breakpoints have been cloned and precisely mapped to the first intron (SM1444) or to 5' flanking sequences (KC1445) of BCL-6 on 3q27, and to the switch region of IgH on 14q32 (20-21). In all rearrangements, the coding region of BCL-6 was left intact whereas the 5' regulatory region, presumably containing the promoter sequences, was either completely removed or truncated. The resultant fusion of BCL-6 coding sequences to heterologous (from other chromosomes) or alternative (within the Bcl-6 locus) regulatory sequences may disrupt the gene's normal expression pattern. A BCL-6 transcript of normal size was detected by Northern blot analysis of DLCL cells carrying either normal or truncated BCL-6. Some of the truncations were in the 5' flanking sequences and would therefore not be expected to generate structurally abnormal transcripts.

Experimental Discussion:

Zinc-finger encoding genes are candidate oncogenes as they have been shown to participate in the control of cell proliferation, differentiation, and organ pattern formation (24). In fact, alterations of zinc-finger genes have been detected in a variety of tumor types. These genes include PLZF (32) and PML (35-38) in acute promyeloctic leukemia, EVI-1 (38-39) in mouse and human myeloid leukemia, TTG-1 (40) in T-cell CLL, HTRX (41-43) in acute mixed-lineage leukemia, and WT-1 (44) in Wilm's tumor. Terminal differentiation of hematopoietic cells is associated with the down-regulation of many Krüppel-type zinc-finger genes. Thus, constitutive expression of BCL-6, caused by chromosomal rearrangements, interferes with normal B-cell differentiation, thereby contributing to the abnormal lymph node architecture typifying DLCL.

Given that DLCL accounts for ~80% of NHL mortality (7), the identification of a specific pathogenetic lesion has important clinicopathologic implications. Lesions in BCL-6 may help in identifying prognostically distinct subgroups of DLCL. In addition, since a therapeutic response can now be obtained in a substantial fraction of cases (7), a genetic marker specific for the malignant clone may be a critical tool for the monitoring of minimal residual disease and early diagnosis of relapse (45).

The gene cloned from chromosomal translocations affecting band 3q27, which are common in DLCL codes for a 79 kD protein that is homologous with zinc-finger transcription factors. In 33% (13/39) of DLCL samples, but no in other types of lymphoid malignancies, the BCL-6 gene is truncated within its 5' noncoding sequences, suggesting that its expression is deregulated. Thus, BCL-6 is a proto-oncogene specifically involved in the pathogenesis of DLCL.

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EXPERIMENTAL DETAIL SECTION III

Introduction:

Non Hodgkin's lymphoma (NHL), the most frequent tumor occurring in patients between the ages of 20 and 40, includes several distinct clinico-pathologic subtypes, among which diffuse lymphoma with a large cell component (DLLC) is the most clinically relevant in terms of morbidity and mortality (1). DLLC include intermediate-grade lymphomas with pure diffuse large- (DLCL), or mixed small- and large-cell (MX-D) histology, as well as high-grade immunoblastic (IMB) lymphoma. These tumors can occur "*de novo* ", accounting for 30-40% of initial NHL diagnosis and, in addition, can represent the final "transformation" stage of follicular lymphomas (FL), small lymphocytic lymphoma and chronic lymphocytic leukemia. Considered together, "*de novo*" and "*post-transformation*" DLLC account for up to 80% of NHL mortality (1).

During the past decade, abnormalities involving proto-oncogenes and tumor suppressor genes have been identified in association with distinct NHL subtypes (2). These genetic lesions represent important steps in lymphomagenesis as well as tumor-specific markers which have been exploited for diagnostic and prognostic purposes (3,4). Examples include alterations of the MYC oncogene in Burkitt lymphoma (BL), and of the BCL-2 and BCL-1 oncogenes in FL and mantle-cell NHL, respectively. With respect to DLLC, several molecular alterations have been detected at variable frequency, but none has been specifically or consistently associated with the disease (2). In this invention the frequency and disease-specificity of BCL-6 (5-10) rearrangements among the principal categories of lymphoproliferative disease, including different NHL subtypes, acute and chronic lymphoid leukemias and multiple myeloma is demonstrated.

Materials and Methods:

Samples of lymphnode biopsies, bone marrow aspirates and peripheral blood were collected by standard diagnostic procedures during the course of routine clinical evaluation in the Division of Surgical Pathology, Department of Pathology, Columbia University. In all instances, the specimens were collected before specific anti-tumor treatment. Diagnoses were based on the results of histopathologic, immunophenotypic and immunogenotypic analysis (11). In all cases, the fraction of malignant cells in the pathologic specimen was at least 70% as determined by cytofluorimetric or immunohistochemical analysis of cell-surface markers or antigen receptor (immunoglobulin heavy chain and T cell receptor β chain) gene rearrangement analysis (11).

Genomic DNA was prepared from diagnostic specimens by cell lysis, proteinase K digestion, phenol-chloroform extraction and ethanol precipitation. For Southern blot analysis, 6 μg of DNA were digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized and transferred to Duralose filters (Stratagene, La Jolla, CA). Filters were then hybridized with the BCL-6-specific Sac 4.0 probe (10) that had been ^{32}P-labelled by the random priming technique. After hybridization, filters were washed in 0.2X SSC (1X SSC = 0.15 M NaCl + 0.015 M sodium citrate / 0.5% sodium dodecyl sulfate) for 2 hours at 60°C and then subjected to autoradiography for 24-48 hours at -80°C using intensifying screens.

All NHL cases were also analyzed for rearrangement of the BCL-2 gene using the previously described probes corresponding to the MBR and MCR regions. Immunophenotypic analysis of immunoglobulin and cell surface marker expression was performed as previously described (11).

Comparisons of histologic subsets with or without BCL-6 rearrangement were made utilizing the method of inferences from proportions (12).

Experimental Results:

The tumor panel (Table 2) used for this study is representative of the major categories of lymphoproliferative disease including NHL, 125 cases, ALL 45, CLL 51 and MM 23. The NHL series was representative of low- 41, intermediate- 45 and high-grade 24 subtypes according to the Working Formulation. Fifteen cases of cutaneous T-cell NHL were also included.

The presence of BCL-6 rearrangements was analyzed by Southern blot hybridization of tumor DNAs using a probe (Sac 4.0) (10) and restriction enzymes (BamHI and XbaI) which, in combination, explore a region of 15.2 Kb containing the 5' portion of the BCL-6 gene (first exon, 7.5 Kb of first intron and 7.4 Kb of 5' flanking sequences) (10). This region was previously shown to contain the cluster of breakpoints detected in NHL. No additional rearrangements were found using probes and restriction enzymes exploring approximately 10kb either 5' or 3' to BCL-6 sequences

The results of this analysis are summarized in Table 2 and representatively shown in Figures 13A-13B. All cases of ALL, CLL and MM showed a normal BCL-6 gene. Eighteen of the 125 NHL cases displayed BCL-6 rearrangements. Among distinct NHL histologic subtypes, rearrangements were detected in 16/45 (35.5%) DLLC and in 2/31 (6.4%) FL ($p < .001$). One of these 2 FL cases showed both follicular and diffuse patterns of growth. Among DLLC, rearrangements were significantly more frequent in DLCL (15/33, 45.4%) than in MX-D (1/10, 10%) ($p < .01$), suggesting that these genetic lesions may be specifically associated with the diffuse large cell component of these tumors. All of the

DLLC cases displaying BCL-6 rearrangements lacked BCL-2 rearrangements which were found in only two 2 DLLC cases. Although cytogenetic data were not available for the panel of tumors studied, the frequency of BCL-6 rearrangements far exceeds that expected for 3q27 aberrations (10-12% in DLLC) (8, 9), suggesting that BCL-6 rearrangements can occur as a consequence of submicroscopic chromosomal aberrations.

In order to determine whether the presence of BCL-6 rearrangements correlated with distinct immunophenotypic features of DLLC, the entire panel was analyzed for expression of immunoglobulin κ and λ light chains, and B cell-associated antigens CD19, CD20 and CD22 (11). As expected, the expression of these markers was variable in the DLLC cases tested. However, no correlation with the BCL-6 rearrangement status was found.

Table 2.

Rearrangements of the BCL-6 gene in lymphoid tumors

TUMOR	HISTOTYPE	REARRANGED/TESTED	%
<u>NHL</u>			
Low grade:	SL	0/10	0
	SCC-F	2*/18	11
	MX-F	0/13	0
Intermediate grade:			
	MX-D	1/10	10
	DLCL	15/33	45
	SCC-D	0/2	0
High grade:			
	IMB	0/2	0
	SNCL	0/22	0
Others:	CTCL	0/15	0
<u>ALL</u>			
	B-lineage:	0/34	0
	T-lineage:	0/11	0
<u>CLL</u>	B-lineage:	0/41	0
	T-lineage:	0/10	0
<u>MM</u>		0/23	0

NHL, non-Hodgkin's lymphoma; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; SL, small lymphocytic; SCC-F, follicular small cleaved cell; MX-F, follicular mixed; MX-D, diffuse mixed cell; DLCL, diffuse large cell; SCC-D, diffuse small cleaved cell; IMB, immunoblastic; SNCL, small non-cleaved cell lymphoma; CTCL, cutaneous T-cell lymphoma. *: one case showed follicular and diffuse growth patterns.

Experimental Discussion:

In this study, BCL-6 rearrangement is established as the most frequent abnormality detectable in DLLC. Previous studies have indicated MYC and BCL-2 rearrangements detectable in 5-20% and 20% of DLLC, respectively (13). Compared to those lesions, which are also commonly associated with Burkitt's lymphoma (MYC) and FL (BCL-2), BCL-6 rearrangements appear to be more disease-specific since they were exclusively found in DLLC with the exception of 2 of 45 FL cases. Considering that one of these two FL cases displayed areas of diffuse histology, it is conceivable that BCL-6 rearrangements may be occasionally associated with atypical FL cases with mixed follicular and diffuse components. The recurrent and specific association between DLLC and structural lesions of a gene coding for a zinc finger-type transcription factor related to several known proto-oncogenes 10 suggests that these abnormalities may play a role in pathogenesis of DLCL.

Among the heterogeneous DLLC spectrum, BCL-6 rearrangements were significantly more frequent in tumors displaying a pure diffuse large cell histology (DLCL) all of which lacked BCL-2 rearrangements. Considering that DLCL can originate both "de novo" and from the "transformation" of FL, and that the latter typically carry BCL-2 rearrangements, results suggest that BCL-6 rearrangements may be specifically involved in the pathogenesis of "de novo" DLLC. This conclusion is consistent with recent findings indicating that other genetic alterations, namely the inactivation of the p53 tumor suppressor gene, may be involved in the transformation of FL to DLLC (14).

The results presented herein have relevant diagnostic and prognostic implications. DLLC represent a heterogeneous group of neoplasms which are treated homogeneously despite the fact that only 50% of patients experience long-term

disease free survival (1). The presence of a marker such as BCL-6 rearrangement identifies a sizable subset of cases with a distinct pathogenesis and, distinct biological behavior.

The pathogenesis of non-Hodgkin lymphoma with a large-cell component (DLLC, including diffuse large-cell, DLCL, diffuse mixed-cell, MX-D, and immunoblastic, IMB) is unknown. The incidence and disease-specificity of BCL-6 rearrangements in a large panel of lymphoid tumors, including acute and chronic lymphoid leukemias (96 cases), various NHL types (125 cases), and multiple myelomas (23 cases) has been tested. BCL-6 rearrangements were found in 16/45 (35.5%) DLLC, more frequently in DLCL (15/33, 45%) than in MX-D (1/10, 10%), in 2/31 (6.4%) follicular NHL, and in no other tumor types. BCL-6 rearrangements represent the first genetic lesion specifically and recurrently associated with DLLC and should prove useful for understanding the pathogenesis as well as for the clinical monitoring of these tumors.

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EXPERIMENTAL DETAIL SECTION IV

Introduction:

Non-Hodgkin lymphomas (NHL) represent one of the most common malignancies associated with human immunodeficiency virus (HIV) infection, and are recognized as an acquired immunodeficiency syndrome (AIDS)-defining condition (1-3). Since their initial observation in 1982 (4), the incidence of AIDS-associated NHL (AIDS-NHL) has been consistently increasing (1, 2), and they now represent the most frequent HIV-associated malignancy in some AIDS risk groups, namely the hemophiliacs (5). Indeed, some estimates project that 10 to 20% of all new NHL cases in the United States may eventually be related to AIDS (6).

AIDS-NHL are almost invariably B-cell derived NHL (1, 2, 7-12). When compared with NHL of similar histology arising in the immunocompetent host, AIDS-NHL display distinctive clinical features, including late stage at presentation, poor prognosis, and the frequent involvement of extranodal sites (1, 2, 7-12). Systemic AIDS-NHL are histologically heterogeneous, and have been initially classified into three distinct categories, including small non cleaved cell lymphoma (SNCCL), large non cleaved cell lymphoma (LNCCL), and large cell-immunoblastic plasmacytoid lymphoma (LC-IBPL) (7, 9). Subsequently, most investigators have agreed to classify LNCCL and LC-IBPL as a single category under the term of diffuse large cell lymphoma (DLCL).

Some progress has been made in elucidating the molecular pathogenesis of AIDS-SNCCL (1-3). AIDS-SNCCL is associated at variable frequency with multiple genetic lesions, including Epstein Barr virus (EBV) infection, c-MYC translocation, RAS gene family mutation, and p53 inactiviation by point mutation and allelic loss (1, 3, 13-25). On the other hand, the pathogenesis of AIDS-DLCL is relatively less defined. EBV infection appears to be the

only genetic lesion associated with a significant fraction of these tumors, particularly with the subset displaying plasmacytoid features, p53 lesions have not been found and c-MYC activation is restricted to a small minority of cases (1-3, 13-25).

Materials and Methods:

Pathologic samples. Biopsy samples of lymph node, bone marrow, peripheral blood, or other involved organs from forty patients with AIDS were collected during the course of standard diagnostic procedures. Thirty-two samples were derived from patients referred to the Department of Pathology, New York University, New York, NY or to the Department of Pathology, Columbia University, New York, NY. Eight samples were derived from patients referred to the Departments of Hematology and Pathology, University of Southern California School of Medicine, Los Angeles, CA. Diagnosis was based on analysis of histopathology, immunophenotypic analysis of cell surface markers, and immunogenotypic analysis of Immunoglobulin (Ig) gene rearrangement (32). In most cases, the fraction of malignant cells in the pathologic specimen was greater than 80%, as determined by cell suspension cytofluorometric or tissue section immunohistochemical analysis of cell surface markers and by Ig gene rearrangement analysis.

DNA extraction and Southern blot analysis. DNA was purified by digestion with proteinase K, "salting out" extraction, and precipitation by ethanol (33). For Southern blot analysis (34), 6 μg of DNA was digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized, transferred to Duralon filters (Stratagene, LA Jolla, CA), and hybridized to probes which had been ^{32}P-labeled by the random primer extension method (35). Filters were washed in 0.2 X SSC (NaCl/Na citrate)/0.5% sodium dodecyl sulphate (SDS) for 2 hours at 60°C and then autoradiographed using

intensifying screens (Quanta III; Dupont, Boston, MA).

DNA probes. Immunoglobulin gene rearrangement analysis was performed using a J_H probe(36) (a gift of Dr. Korsmeyer) on *Hind*III, *Eco*RI, and *Bam*HI digests. The organization of the *BCL-6* locus was investigated by hybridization of *Xba*I, *Bam*HI, and *Bgl*II digested DNA to the human *BCL-6* probe Sac4.0 (26-27). In selected cases, a second probe representative of the *BCL-6* locus, Sac0.8, was also used. The organization of the c-*MYC* locus was analyzed by hybridization of *Eco*RI and *Hind*III digested DNA to the human c-*MYC* locus was analyzed by hybridization of *Eco*RI and *Hind*III digested DNA to the human c-*MYC* probe MC413RC, representative of the third exon of the c-*MYC* gene (37). The presence of the EBV genome was investigated with a probe specific for the EBV termini (5.2 Kb *Bam*HI-*Eco*RI fragment isolated from the fused *Bam*HI terminal fragment NJ-het) (38).

<u>Experimental Results</u>:

Forty cases of systemic AIDS-NHL were studied, including 13 SNCCL and 24 DLCL (8 LNCCL and 16 LC-IBPL). In addition, three cases of CD30+ lymphomas, which have been sporadically reported in AIDS (39), were also included. All cases displayed a predominant monoclonal B-cell population as determined by Ig gene rearrangement analysis.

Analysis of *BCL-6* rearrangements. The *BCL-6* gene contains at least 9 exons spanning approximately 26 Kb of genomic DNA (27). Sequence analysis has shown that the first exon is non-coding and that the translation initiation codon is located within the third exon (27). Rearrangements of *BCL-6* can be detected by Southern blot analysis using a probe (Sac4.0) and restriction enzymes (*Bam*HI and *Xba*I) which, in combination, explore a region of 15.2 Kb containing the 5' portion of the *BCL-6* gene (27) (Figures 14A-14C). This same region was previously shown to contain the cluster of

chromosomal breakpoints detected in NHL of the immunocompetent host (27, 29). Cases showing an abnormally migrating band in only one digest were further studied by hybridizing the Sac4.0 probe to additional digests (*Bgl*II) or, alternatively, by hybridizing *Bam*HI and *Xba*I digests to a probe (Sac0.8) derived from the *BCL-6* first intron, which, being located 3' of the breakpoint cluster, explores the reciprocal chromosome 3 (Figures 14A-14C). Only cases showing abnormally migrating bands with two restriction enzymes and/or two probes were scored as rearranged.

Rearrangements of *BCL-6* were detected 5/24 AIDS-DLCL (20.8%), both in the LNCCL (2/8; 25%) and in the LC-IBPL (3/16; 18.7%) variants (Table 3 and Figures 14A-14C). All cases of AIDS-SNCCL and CD30+ lymphomas displayed a germline *BCL-6* locus. The location of the breakpoints detected in AIDS-HNL corresponds to the pattern most commonly observed in DLCL of the immunocompetent host.

Table 3.

Frequency of BCL-6 rearrangements in AIDS-NHL

SNCCL[a]	DLCL[b]		CD30+NHLc
	LNNCL	LC-IBPL	
0/13	2/8	3/16	0/3

[a]: SNCCL, small non cleaved cell lymphoma

[b]: DLCL, diffuse large cell lymphoma. The DLCL included in the panel can be further distinguised into two subgroups (LNCCL, large non cleaved cell lymphoma; and LC-IBPL, large cell immunoblastic-plasmacytoid lymphoma) as previously reported (7,9).

c: Non-Hodgkin lymphoma expressing the CD30 cell surface antigen (39).

Other genetic lesions. The other genetic lesions

investigated in the panel of AIDS-NHL included infection by EBV of the tumor clone, activation of the c-*MYC* and *RAS* proto-oncogenes, and inactivation of the p53 tumor suppressor gene. The experimental strategies used to investigate these lesions have been described in detail elsewhere (13, 45, 40). For some of the

cases, the molecular characterization of these genetic lesions have been previously reported (13, 14, 41); for the other cases, it has been assessed in the course of this study.

EBV infection was assessed by Southern blot hybridization using a probe representative of the EBV termini (38) which allows to analyze clonality in EBV-infected tissues (23) (Figures 16A-16C). A monoclonal infection was detected in 5/13 (38%) SNCCL, 17/24 DLCL (71%) [3/8 (37.5%) LNCCL and 14/16 (87.5%) LC-IBPL], and 3/3 (100%) CD30+ cases.

Rearrangements of c-*MYC* were tested by hybridizing *Hind*III and *Eco*RI digested DNAs with a probe representative of c-*MYC* exon 3(41) (Figures 16A-16C). Rearrangements were present in 13/13 SNCCL (100%), 5/24 (20.8%) DLCL [2/8 (25%) LNCCL and 3/16 (18.7%) LC-IBPL], and 2/3 CD30+ cases.

Mutations of *p53* and *RAS* were analyzed by a two step strategy. Single strand conformation polymorphism (SSCP) analysis was applied to *p53* exons 5 through 9 (in 29 cases) or *p53* exons 5 through 8 (in 6 cases) and to N-, K-, and H-*RAS* exons 1 and 2 (in 29 cases); cases displaying an altered electrophoretic pattern by SSCP were further studied by DNA direct sequencing of the PCR product. *p53* mutations were scored in 8/13 (61.5%) SNCCL, but in none of the DLCL tested (0/22). Finally, *RAS* activation by point mutation was positive in 3/13 (23%) SNCCL and in 1/16 (6%) DLCL tested.

The molecular features of the cases displaying *BCL-6* rearrangements are listed in Table 4. Overall, *BCL-6* rearrangements were detected both in the presence and in the absence of clonal EBV infection of the tumor, whereas c-*MYC* alterations and *p53* mutations were consistently absent in the cases displaying *BCL-6* rearrangements.

Table 4.

Molecular features of AIDS-DLCL[a]

PATIENT	HISTOL.[b]	CLONALITY	BCL-6	EBV	c-MYC	p53	RAS
DK782	LNCCL	+	+	-	-	-	-
DK1178	LNCCL	+	+	-	-	-	-
DK1028	LNCCL	+	-	-	-	-	-
DK3973	LNCCL	+	-	+	-	-	-
DK773	LNCCL	+	-	-	+	-	-
RDF834	LNCCL	+	-	+	+	-	-
DK1452	LNCCL	+	-	-	-	-	-
DK64	LNCCL	+	-	+	-	-	+
DK771	LC-IBPL	+	+	+	-	-	-
K827	LC-IBPL	+	+	+	-	-	-
DS16	LC-IBPL	+	+	+	-	-	ND
DK3537	LC-IBPL	+	-	+	+	-	-
DK3357	LC-IBPL	+	-	+	-	-	-
DK63	LC-IBPL	+	-	+	-	-	-
DK1446	LC-IBPL	+	-	+	+	-	-
DK3479	LC-IBPL	+	-	-	-	-	-
DK2092	LC-IBPL	+	-	+	-	-	-
DS17	LC-IBPL	+	-	-	-	-	ND
DS45	LC-IBPL	ND	-	+	-	-	ND
DS46	LC-IBPL	+	-	+	+	-	ND
DS93	LC-IBPL	+	-	+	-	-	ND
DS136	LC-IBPL	+	-	+	-	-	ND
DS155	LC-IBPL	+	-	+	-	-	ND
DS165	LC-IBPL	+	-	+	-	-	ND

a: The results of the analysis of EBV, c-MYC, p53 and RAS of some of these cases have been previously reported (14, 15, 41).

b: LNCCL, large non cleaved cell lymphoma; LC-IBPL, large cell-immunoblastic plasmacytoid lymphoma

c: ND, not done

Experimental Discussion:

Diffuse large cell lymphoma (DLCL) represents the most frequent type of AIDS-NHL in the HIV-infected adult (8). Despite its epidemiologic relevance, the molecular pathogenesis of these tumors is largely unclarified (3). Analysis of the genomic configuration of *BCL-6* in a panel of AIDS-NHL indicates that *BCL-6* rearrangements are involved in approximately 20% of AIDS-DLCL, whereas they are consistently negative in AIDS-SNCCL. In this respect, *BCL-6* rearrangements may be considered the first identified genetic lesion specific for the DLCL type among AIDS-NHL. *BCL-6* rearrangements are present in both subgroups of DLCL, i.e. LNCCL and LC-IBPL, and occur both in the absence and in the presence of EBV infection of the tumor clone (Table 4). On the other hand, *BCL-6* rearrangements were never detected in AIDS-DLCL carrying c-*MYC* alterations (Table 4).

The molecular pathway leading to AIDS-SNCCL involves c-*MYC* rearrangements, *p53* mutations, and EBV infection in 100%, 60%, and 40% of the cases, respectively (13-26). The presence of somatic hypermutation in the immunoglobulin variable regions utilized by AIDS-SNCCL points to chronic antigen stimulation as an additional mechanism in the development of these tumors. The second genetic pathway is associated with AIDS-DLCL, involves EBV in the large majority of cases, as well as c-*MYC* and/or *BCL-6* rearrangements in a fraction of cases (13-26). These distinct pathogenetic mechanisms correlate with a number of clinical features which distinguish AIDS-SNCCL from AIDS-DLCL, including different age of onset and different CD4 counts at the time of lymphoma development (1,2,8).

Results suggest that the frequency of *BCL-6* rearrangements in AIDS-DLCL is significantly lower than that in DLCL in the immunocompetent host, where *BCL-6* rearrangements occur in more than 40% of the cases. It is possible that the

genetic pathogenesis of these two groups of tumors is different, and that the molecular mechanisms active in AIDS-DLCL are characterized by a higher degree of heterogeneity. Among DLCL in the immunocompetent host, *BCL-6* rearrangements are associated with distinct clinical features, including the extranodal origin of the lymphoma and the lack of bone marrow involvement. In addition, the presence of this rearrangement appears to represent a favorable prognostic marker.

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EXPERIMENTAL DETAIL SECTION V

Introduction:

The group of diffuse lymphomas with a large cell component (DLLC), including diffuse mixed, immunoblastic, and large cell subtypes, and the group of follicular lymphomas, each comprise about 40 per cent of non-Hodgkin's lymphomas (NHL) in this country (1). Together, the incidence of NHL is increasing at 3 to 4 per cont a year, a rate second only to that of malignant melanoma and lung cancer in women (2). Despite significant advances in treatment, approximately half of patients with DLLC will succomb to their disease, although "high risk" individuals may successfully be treated by intensive chemotherapy and radiotherapy regimens including autologous bone marrow transplantation (3-7). The formulation of prognostic models allow clinical trials to be directed toward groups of patients with different risks for failure after conventional treatment (5).

Cytogenetic studies as well as molecular genetic analysis of alterations involving proto-oncogenes and tumor suppressor genes have provided insights into the pathogenesis of NHL, and have also contributed diagnostic and prognostic markers (8,9). Examples include rearrangements of the BCL-2 gene at 18q21 observed in up to 85 per cent of follicular lymphomas, the BCL-1 gene at 11q13 rearranged in intermediate differentiation NHL, and the MYC gene, perturbed in Burkitt's lymphoma (8,9). While no recurring genetic abnormality has been specifically associated with diffuse large cell lymphoma, rearrangement of BCL-2 has been observed in 20 to 30 per cent of cases, where it has been associated with decreased overall or disease free survival (10-12). Chromosomal translocations including those involving the MYC proto-oncogene, while noted in DLLC, were not as prognostically significant as other recurring chromosomal abnormalities (8,13).

BCL-6 (14-19) rearrangement is found to denote a subset of DLLC characterized by extranodal presentation and a favorable clinical outcome. These results indicate that, in concert with other clinical features, this molecular marker may be utilized as a prognostic indicator at the time of diagnosis.

Materials and Methods:

This study was comprised of 102 cases of DLLC studied at diagnosis with documented clonal rearrangement of the IGH gene and DNA available for further analysis, derived form 229 DLLC serially ascertained over a nine year period. Excluded were 127 cases studied at relapse, T cell DLLC, or cases for which no DNA was available. For this study, DLLC was defined as lymphomas of diffuse large cleaved, non-cleaved, immunoblastic, or mixed subtype, according to the International Working Formulation (20) as classified by a hematopathologist (DCS or DF). Cytogenetic analysis was attempted on each of the specimens as previously described (21). For detection of BCL-6 rearrangements, DNA from each case was digested with BamHI and XbaI and subjected to Southern blot analysis utilizing a 4kb Sac1-Sac1 fragment of the BCL-6 gene as a probe (19). Cases which did not yield metaphases for karyotypic analysis were also analyzed for rearrangement of the MBR and MCR breakpoint regions of the BCL-2 gene, as previously described (11). Aggregate descriptions of 47 of the cases in the current series were included in prior reports of cytogenetic abnormalities in DLLC (11, 13, 14). A detailed molecular analysis of 8 cases (nos. 352, 755, 1098, 1254, 1403, 1444, 1445) demonstrating BCL-6 rearrangement has been reported separately (19).

For each case, clinical data were compiled as previously described (22). Stage was assessed according to the modified Ann Arobr criteria (25). For the purposes of separate evaluation of number of extranodal sites of

disease, radiographs or pathologic involvement of these sites were scored. In the quantitation of extranodal sites of disease as a prognostic variable, bone marrow, but not splenic involvement was scored, in accord with the International Prognostic Index (5).

Clinical endpoints including complete response and freedom from progression were defined as previously described (3). Of 102 patients with DLLC genetically analyzed prior to cytotoxic treatments, 93 received systemic chemotherapy. Nine patients with early stage disease were treated by surgical resection and/or radiation therapy. All patients were treated with curative intent. Chemotherapy treatments were classified into three groups: NHL-4, CHOP and BACOP (1st generation); m-BACOD, NHL-7 (2nd generation); MCOP_B, NHL-9, NHL-14, NHL-15, L-20 (3rd generation)(4,24,29). Eight patients expired before completion of therapy, with incomplete staging evaluations, or of infectious complications during or shortly after treatment. These cases were considered not valuable for the determination of remission status, but were included in the analysis of overall survival and freedom from progression. One patient was judged to be a complete remission which was confirmed by autopsy after expiration due to infectious complications 3 weeks after completion of protocol treatment. All deaths, regardless of cause were considered as endpoints in the analysis of overall survival. Median survival was determined by the method of Kaplan and Meler (30). Analysis of correlations between gene rearrangements and clinical features were performed utilizing Fisher's exact test (13). Means were compared utilizing two sample 't-tests. Univariate comparisons of survival and duration free from progression were made by log rank test. Survival and freedom from progression estimates are quoted with confidence intervals (CI) given in parentheses. Multivariate analysis was performed utilizing the Cox regression model (31). Stepwise multiple logistic regression was sued in the multivariate analysis of factors

prognostic for achieving a complete response. For all statistical analyses, a P<0.05 based on a 2-sided test was considered significant.

Experimental Results:

Of 102 cases of DLLC studied at diagnosis, 23 demonstrated BCL-6 rearrangement, 21 demonstrated t(14;18) or rearrangement of BCL-2, and 58 demonstrated no evidence of either BCL-6 or BCL-2 rearrangement. Representative results of hybridization analysis for rearrangement of BCL-6 are depicted in Figures 17A-17B. The clinical characteristics of groups according to BCL-6 or BCL-2 rearrangement are summarized in Table 5. The histologic subtypes and clinical features of the BCL-6 rearranged cases are shown in Table 6.

The key to Table 6 is as follows: Underlining signifies site from which biopsy was performed. Histology: DLC = diffuse large cell; IMB - immunoblastic; DML = diffuse mixed lymphoma; LDH = lactate dehydrogenase in units/ml; (B) = bulky disease (> 8 cm or 1/3 thoracic diameter); CHOP = cyclophosphamide, daunorubicin, vincristine, prednisone; MACOPB = methotrexate, daunorubicin, cyclophosphamide, vincristine, prednisone, bleomycin; MBACOD = same drugs as MACOPB with dexamethasone instead of prednixone and drugs in different schedule; PrCyBom - drugs of MACOPB plus cytosine arabinoside, etopiside, methotrexate; L-20 - vincristine, cyclophosphamide, methotrexate, daunorubicin, prednisone, cytosine arabinoside, 1-asparaginase, BCNU, 6-mercaptopurine, dactinomycin; 1-20 includes randomization to autologous transplantation; NHL-7 = CHOP plus methyl GAG, etoposide(36), NHL-14 = short course PrCyBom; NHL-15 = high dose daunorubicin, vincristine, cyclophosphamide(29); RT = radiation therapy; SURG' = surgery CR = complete response; sCR = surgical complete response (all evaluable disease resected); PR = partial response; "+" = alive at last follow-up; e = expired; rel

= relapse; NE = not evaluable; * = Patient 1445 had a history of low grade NHL of eyelid 7 years earlier, treated by radiotherapy. # Skin involvement of patient 252 was not noted in a prior report (14) and patient 1445 had a history of low grade NHL of eyelid 7 years earlier, treated by radiotherapy.

While each of the BCL-6 rearranged cases was classified as a DLLC, the range of morphologies included diffuse large cell (cleaved and non-cleaved), and less frequently, immunoblastic, or mixed histologies. Extensive necrosis and extranodal extension were common histologic features, and were present in one of two cases of BCL-6 rearrangement which did not show clinical evidence of extranodal disease.

The BCL-6 rearranged cases had a mean age of 64.1 years at presentation and a high frequency of extranodal involvement by disease; 19 of 23 cases had stages IE, IIE, IIIE or stage IV desease, compared to 48 of 79 of BCL-6 germline cases (p+0.07). Extranodal sites included muscle or subcutaneous tissues (6 cases), stomach (5 cases), lung or pleura (5 cases), skin, breast, bowel, thyroid, pancreas, or kidney, as assessed by biopsy or radiographic abnormalities which improved after chemotherapy. Of the 7 cases with state IE or IIE decrease, 5 were primary extranodal lymphomas, while 2 were extranodal extensions from a primary nodal site. Two cases were primary splenic lymphomas. In two cases, there was only peripheral adenopathy. Compared to BCL-6 germline cases, there was no significant difference in the proportion of BCL-6 rearranged cases with stage IV disease. Bone marrow involvement was observed in 15 of 75 BCL-6 germline cases biopsied, compared to only 1 of the 23 stage IV BCL-6 rearranged cases (P=0.1).

All but one of the 23 patients with BCL-6 rearrangement at the time of diagnosis received anthracycline-containing chemotherapy. This patient remained free of disease eight

years after resection of a primary splenic large cell lymphoma. At median follow-up in excess of two years, 21 of the 23 patients with BCL-6 rearrangement survived; the actuarial survival was 91 per cent (CI 80 per cent to 100 per cent). Two patients expired during or immediately following treatment; an autopsy in one case revealed no evidence of lymphoma. This patient, and 19 others were judged to have achieved a complete remission after treatment. Two patients relapsed with recurrent disease in the lung and two patients had persistent subcutaneous masses. One of the relapse patients (case 295) went on to autologous transplanation and remains free of disease 78 months post-transplant.

With respect to known prognostic variables, the proportion of the *BCL-6* rearranged cohort with LDH > 500 U per liter was similar to the proportion of the BCL-6 germline DLLC (3/23 versus 13/79; P=0.99). Five of 23 cases of DLLC with BCL-6 rearrangement demonstrated bulky disease, compared to 35 of 79 cases without BCL-6 rearrangement (P=0.1). The proportion of cases with "limited stage" (I, IE, II, or IIE) disease was comparable in the cohorts with and without BCL-6 rearrangement (Table 5).

Table 5.

Characteristics of 102 cases of DLLC

	BCL-6+	BCL-6- BCL2-	BCL-6+ BCL2+
n=	23	58	21
Mean age (years)	64.1	52.7	62.8
Mean lactate dehydrogenase (U/ml)	405	331	389
Mean extranodal sites	1.6	.93	.81
Bone marrow involvement	1/23	8/54	7/21
Stage			
I(IE)	3(1)	3(3)	0
II(IIE)	7(6)	22(6)	5
III(IIIE)	2(1)	8(2)	4
IV	11	25	12
Histology			
Diffuse large cell	20	53	19
Diffuse mixed	1	2	1
Immunoblastic	2	3	1
Treatment			
1st generation chemo.	12	16	10
2nd generation chemo.	1	10	3
3rd generation chemo.	9	24	8
other	1	8	0
Complete Remission	20/23	35/50	15/21
Rate	86%	70%	71%
Projected survival at 36 months	91% (CI 80%-100%)	59% (CI 44%-74%)	46% (CI 21%-72%)
Projected freedom from progression at 36 months	82% (CI 66%-98%)	56% (CI 43%-70%)	31% (CI 8%-53%)

Table 6.

Clinical features of 23 cases of DLLC with BCL6 rearrangement.

Case Number	Age/ Sex	Stage	Extranodal Site	Histology	LDH (Bulk)	Treatment	Clinical Outcome
102	66/F	IIS	spleen	DLC	3624	SURG, CHOP	CR, 96+
147	61/F	IS	spleen	DLC	365 (B)	SURG, RT	sCR, 101+
252	54/M	IV	spleen, skin#	DLC	126	MACOPB	CR, 88+
278	68/M	IV	pleura, iliac mass	DLC	235 (B)	MACOPB	PR, 6e
295	46/M	IV	lung	DLC	179	MACOPB,L-20	CR, rel, 97+
352	53/F	IV	stomach, liver, spleen, small bowel, pleural effasion	IMB	775 (B)	MACOPB	CR, 81+
470	74/F	IV	lung	DLC	224	CHOP	CR, 30+
534	70/F	IIES	spleen, mass involving pancreas	DLC	278 (B)	CHOP	CR, 80+
763	79/F	IIE	stomach	DLC	196	CHOP	CR, 60+

970	75/M	IV	kidney, stomach	DLC	240	NHL-14	CR, 4e
1020	60/M	IIIE	tonsil, pancreas	DLC	303	CHOP	CR, 100+
1056	63/M	IIE	<u>stomach</u>	DLC	213	SURG, MBACOD	CR, 100+
1058	59/M	IIE	<u>axillary mass involving breast</u>	DLC	206	PrCyBom	CR, 37+
1098	74/F	IV	<u>subcutaneous masses</u>	DML	181	RT, CHOP	PR, 36+
1189	71/M	IV	<u>subcutaneous masses</u>	DLC	330	CHOP	PR, 21+
1254	74/F	IIE	<u>thyroid</u>	DLC	196	CHOP/RT	CR, 27+
1264	76/F	IV	<u>Lung</u>, liver spleen, kidney	DLC	234	CHOP	CR, rel 27+
1299	50/M	IE	<u>deltoid mass</u>, bone	DLC	529 (B)	PrCyBom	CR, 16+
1363	47/M	III	NONE	DLC	129	NHL-15	CR, 16+
1403	62/M	I	NONE	IMB	222	CHOP, RT	CR, 11+

1407	71/M	IIE	<u>stomach</u>	DLC	206	SURG, CHOP	CR, 12+
1444	70/F	IV	<u>lung</u>	DLC	150	CHOP	CR, 14+
445	63/F	IV	neck mass involving muscle*, <u>bone marrow</u>	DLC	174	NHL-15	CR, 8+

Multivariate analysts of clinical outcome. The median duration free from progression of disease was not reached in the BCL-6 rearranged DLLC compared to 70 months for BCL-6 germline cases, regardless of BCL-2 status (P=0.009) (Figure 18A). Projected freedom from progression at 36 months was 82% (CI 66%- 98%) and 49% (CI 37%-60%), respectively. Multivariate analysis revealed that four variables, BCL-6 status, stage IV disease, bulk of disease, and LDH (log transformed) were the most powerful prognostic indicators for freedom from progression (Table 7). Multivariate analysis of survival demonstrated that bulk, LDH, BCL-6 status, and stage IV disease were the most useful predictors of overall survival (P=0.01, P=0.02, P=0.02, P=0.05, respectively).

Table 7

Multivariate analysis of freedom from progression

Variables selected into Cox regression model	Relative Risk	P value (Wald chi square)
BCL-6 rearranged	0.18 (CI).04-.78)	0.007
Bulky disease	2.4 (CI 1.3-7.4)	0.01
Stage IV disease	2.1 (CI 0.98-5.2)	0.03
LDH (log transformed)	1.6 (CI 1.1-3.9)	0.05

The prognostic value of BCL-6 gene status was compared to risk variables calculated according to the International Prognostic Index[5], including serum LDH level, stage, performance status, and number of extranodal sites. A cox regression analysis confirmed the independent prognostic value of BCL-6 gene status; patients with BCL-6 rearrangement had a relative risk (RR) of dying of .09 (CI

.02 to .42) compared to patients without BCL-6 rearrangement, controlling for the other prognostic variables in the model (P=0.002).

When cases were considered with respect to BCL-2 status, the BCL-2 rearranged cases demonstrated a trend for a decreased survival compared to BCL-2 germline cases, regardless of BCL-6 status (P=0.12). When BCL-6 and BCL-2 status were considered together (Figure 18B), BCL-6 rearranged cases demonstrated a projected actuarial survival at 36 months of 91% (CI 80%-100%) compared to 59% (CI 44%-74%) for the BCL-6 germline/BCL-2 germline cohort, and 46% (CI 21%-72%) for the BCL-2 rearranged cohort. While the logrank test between these three cohorts demonstrated a difference in survival (P=0.02, Figure 18B), the major factor driving the significant summary P value was the better survival of the BCL-6 rearranged cohort. The projected freedom from progression at 36 months was 82% (CI 66%-98%), 56% (CI 43%-70%) and 31% (CI 8%-53%) for the three groups. Median follow-up for survivors was two years. BCL-2 rearrangement did not emerge as an independent prognostic marker in the multivariate analysis of survival or freedom from progression.

There was also no prognostically significant effect of generation of chemotherapy treatment on survival, or freedom from progression (P=0.95, 0.21, respectively). There was a trend for a higher complete response rate among the BCL-6 rearranged cohort (Table 5, P=0.1), although logistic regression revealed that only the clinical parameters LDH, stage IV, and bulk of disease were independent predictors of response.

Relationship between BCL-6, BCL-2, and 8q24 rearrangements. Of the 79 cases which lacked BCL-6 rearrangement, 21 demonstrated t(14;18)(q32;q21) or rearrangement of BCL-2 by molecular analysis. These cases were characterized by an older age at diagnosis, but were similar to the larger

cohort of BCL-2 negative, BCL-6 negative cases with respect to LDH, and distribution of histologies (Table 6).

Nine cases of DLLC demonstrated t(8;14)(q24;q32). Three of these biopsies were from extranodal sites including liver, bone and soft tissue. Two additional cases were splenic lymphomas. In two cases, t(8;14) bearing DLLC also demonstrated BCL-6 rearrangement. There was no impact on survival of the t(8;14) in DLLC with or without BCL-6 rearrangement. The two cases of t(8;14) with co-incident BCL-6 rearrangement did not show evidence of histologic transformation or other unusual histologic features. One of these cases (no. 147) was the single case treated by splenectomy and radiation therapy alone. The second case was the single BCL-6 rearranged case successfully salvaged by autologous transplantation.

Cytogenetic features, including the relationship between 3q27 and BCL-6 rearrangement. Of the 65 DLLC with karyotypic abnormalities, 14 demonstrated translocations and one a deletion affecting band 3q27; only 11 among these 15 cases showed rearrangement of BCL-6 Five cases with apparently normal chromosomes 3 demonstrated BCL-6 rearrangements by DNA analysis.

Experimental Discussion:

As a group, DLLC are among the most common forms of NHL seen in this country (1). These tumors have not, however, been associated with a characteristic genetic abnormality (8). Seen in the vast majority of follicular lymphomas, t(14;18)(q32;q21) or its molecular equivalent, BCL-2 rearrangement, have been observed in 20 to 30 per cent of DLLC (8). In such cases, the t(14;18) may reflect a follicular origin of these tumors. The recognition of translocations involving 3q27 and the sites of IG genes, 14q32, 22q11, and 2p12, in predominantly diffuse NHL led to the molecular cloning of BCL-6 (14-19). While not unique to diffuse large cell lymphomas, translocations affecting 3q27 were observed only in 7 of > 200 cases off follicular NHL with abnormal karyotype reported in catalog of chromosome abnormalities in cancer (32). Of 28 cases of follicular NHL analyzed in a prior study, none demonstrated rearrangement of BCL-6 (19). BCL-6 rearrangement was established as the most common genetic lesion specific to DLLC at the time of diagnosis.

Unlike 18q21 translocations in NHL which, to date only have involved IG gene loci as reciprocal partners, 3q27 translocations demonstrated a marked promiscuity of rearrangement partners. In addition to the sites of the IG genes, reciprocal translocations involving the 3q27-29 region with at least 12 other loci; a total of 79 DLLC with 3q27 translocations has been demonstrated.

Since 4 tumors in the current series with documented 3q27 aberrations did not reveal BCL-6 rearrangement with the probe used in this study, the true frequency of BCL-6 rearrangement in DLLC at diagnosis may be higher than the 23 per cent rate reported here. Additional breakpoints may be documented outside the recognized break cluster region of BCL-6 (19), in neighboring genes such as EV-1 (34), or in other genes not yet described. Such molecular

heterogeneity is not unique in NHl; seemingly identical chromosomal translocations have been shown to demonstrate a diversity of breakpoints possibly involving different genes (35).

The frequent occurrence of BCL-6 rearrangement in DLLC characterized by extranodal involvement represents one of the few genetic markers for this subset of lymphoma (8). Rearrangements of BCL-1, BCL-2, or BCL-3 have been documented infrequently in extranodal lymphomas (36-38), while 5 of 12 gastric lymphomas in one series demonstrated MYC (8q24) rearrangement (38). The current series did not confirm the association between 8q24 rearrangement and gastric lymphoma, although t(8;14) was seen in five cases of extranodal lymphoma, one of which also showed BCL-6 rearrangement. The proportion of BCL-6 rearranged cases with stages IE, IIE, IIIE, or IV disease was higher than the proportion of BCL-6 germline DLLC; in the latter group, stage IV disease was more commonly due to bone marrow involvement. Whether this association with extranodal involvement of disease reflects an effect of the primary deregulation of BCL-6 or "secondary" genetic events associated with tumor progression (8,21) is unclear. The observation of t(3;22), t(2;3), or t(3;14) as solitary cytogenetic abnormalities in some tumors (14,15), is consistent with a primary pathogenetic role for this translocation.

While this analysis and two other reports did not confirm the very short survival of BCL-2 rearranged DLLc initially reported (10, 12, 13, 39), the BCL-2 rearranged DLLC did demonstrate a trend for a poorer overall survival. The finding of a favorable prognosis for the subset of stage IE-IIE extranodal DLLC with BCL-6 rearrangement is consistent with prior reports of a good prognosis associated with localized extranodal large cell NHL treated with surgery or radiotherapy (40). Extranodal involvement in advanced stage disease, noted in the majority of the

BCL-6 rearranged cases, has generally been considered a poor prognostic factor in large series of DLLc, although the negative impact of this feature was most evident when combined with other adverse indicators such as bulk, high LDH, or low performance status (5, 22, 41). In contrast, bone marrow involvement, observed in 22 percent of DLLC, and considered an extranodal site in the International Prognostic Index (5), was rare in the BCL-6 rearranged cohort. The favorable treatment outcome of the BCL-6 cohort, must also be tempered by the observation of relapse or residual disease in 3 of the patients still alive. An additional relapse case remains in remission 6 years after "salvage" autologous transplantation.

The BCL-6 rearranged cohort of DLLC also possessed other clinical markers of favorable prognosis; although comparable with respect to LDH and proportion with stage I-IIIE disease, the proportion of cases with bulky disease or bone marrow involvement was lower in the BCL-6 rearranged cohort. Multivariate analysis suggested, however, that BCL-6 gene rearrangement added independent prognostic power when analyzed together with clinically-derived variables of the International Prognostic Index (5). This observation is illustrated by case 352, which displayed both BCL-6 rearrangement as well as clinical features consistent with a high level of risk in the International Index (elevated LDH, extensive extranodal involvement, low performance status, and stage IV disease), but who attained a durable remission.

Because of issues of toxicity versus efficacy of autologous bone marrow transplantation or peripheral stem cell rescue, the identification of both favorable and unfavorable prognostic markers offers the potential to stratify treatment approaches to DLLC based on risk groups (4-7, 22, 41). The probability of treatment failure remains as high as 25-40 per cent for the most favorable subsets of DLLC based on current prognostic models, highlighting the need

for genetic or other prognostic markers (5). In addition to its potential diagnostic and prognostic applications, the further identification of BCL-6 breakpoint regions offers the opportunity to develop new polymerase chain reaction-derived measures of minimal residual disease (43). The availability of BCL-6 rearrangement as a new molecular marker of large cell lymphoma constitutes a potentially important clinical tool in the management of patients with this desease.

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Experimental Detail Section VI

Introduction

The *bcl*-6 proto-oncogene encodes a POZ/Zinc finger transcriptional repressor expressed in germinal center (GC) B and T cells and required for GC formation and antibody affinity maturation. Deregulation of *bcl*-6 expression by chromosomal rearrangements and point mutations of the *bcl*-6 promoter region are implicated in the pathogenesis of B-cell lymphoma. The signals regulating *bcl*-6 expression are not known. Here the antigen receptor activation leads to BCL-6 phosphorylation by mitogen-activated protein kinase (MAPK) is shown. Phosphorylation, in turn, targets BCL-6 for rapid degradation by the ubiquitin/proteasome pathway. These findings indicate that BCL-6 expression is directly controlled by the antigen receptor via MAPK activation. This signaling pathway may be crucial for the control of B-cell differentiation and antibody response and has implications for the regulation of other POZ/Zinc finger transcription factors in other tissues.

The *bcl*-6 proto-oncogene was identified by virtue of its involvement in chromosomal translocations in diffuse large cell lymphoma (DLCL), the most common form of non-Hodgkin's lymphoma (NHL) (Baron et al., 1993; Kerckaert et al., 1993; Ye et al., 1993; Miki et al., 1994). Subsequent studies have demonstrated that rearrangements of the *bcl*-6 gene can be found in 30-40% of DLCL and in a minority (5-10%) of follicular lymphoma (FL) (Bastard et al., 1994; LoCoco et al., 1994; Otsuki et al., 1995). These rearrangements juxtapose heterologous promoters, derived from other chromosomes, to the *bcl*-6 coding domain, causing its deregulated expression by a mechanism called promoter substitution (Ye et al., 1995; Chen et al., 1998). The 5' noncoding region of the *bcl*-6 gene can also be altered by somatic point mutations that are detectable, independent of rearrangements, in ~70% DLCL, 45% FL and AIDS-associated

NHL (Migliazza et al., 1995; Gaidano et al., 1997). Taken together, rearrangements and mutations of the *bcl-6* promoter region represent the most frequent genetic alteration in human B-cell malignancies, suggesting that they may be important for tumorigenesis (Dalla-Favera et al., 1996).

The BCL-6 protein is a nuclear phosphoprotein belonging to the POZ/Zinc finger (ZF) family of transcription factors (Kerckaert et al., 1993; Ye et al., 1993; Miki et al., 1994). It contains six *Krüppel*-type carboxy-terminal zinc finger (ZF) motifs that have been shown to recognize specific DNA sequences in vitro (Chang et al., 1996; Seyfert et al., 1996) and an amino-terminal POZ motif (Albagli et al., 1995) shared by various ZF molecules including the *Drosophila* developmental regulators *Tramtrack* and *Broad-complex* (Harrison and Travers, 1990; DiBello et al., 1991), the human KUP (Chardin et al., 1991), ZID (Bardwell and Treisman, 1994) and PLZF (Chen et al., 1993) proteins as well as by POX viruses (Koonin et al., 1992) and the actin-binding *Drosophila* oocyte protein *Kelch* (Xue and Cooley, 1993). BCL-6 functions as a potent transcriptional repressor by binding to its DNA target sequence (Deweindt et al., 1995; Chang et al., 1996; Seyfert et al., 1996).

BCL-6 is an important regulator of lymphoid development and function. In the B-cell lineage, the BCL-6 protein is found only in B cells within germinal centers (GC), but not in pre-B cells or in differentiated progeny such as plasma cells. In the T-cell lineage, BCL-6 protein is detectable in cortical thymocytes and in $CD4^+$ T cells within GC as well as scattered in the perifollicular area (Cattoretti et al., 1995; Onizuka et al., 1995; Allman et al., 1996). Mice deficient in BCL-6 display normal B-cell, T-cell and lymphoid organ development, but have a selective defect in T-cell-dependent antibody responses because of the inability of follicular B cells to proliferate and form GC

(Dent et al., 1997; Ye et al., 1997). In addition, BCL-6-deficient mice develop an inflammatory response in multiple organs characterized by infiltration of eosinophils and IgE-bearing B lymphocytes typical of a Th2-mediated inflammatory response. These phenotypes may be explained by the ability of BCL-6 to bind the STAT-6 DNA-binding site and repress transcription activated by STAT-6, the main nuclear effector of IL-4 signaling (Dent, et al., 1997; Ye et al. 1997).

The expression and requirement of BCL-6 during GC formation and its alteration in GC-derived lymphoma suggest that BCL-6 may be a key regulator of GC development and antibody-mediated immune response. Toward the elucidation of the signals that regulate GC expression, we report here the identification of a signaling pathway by which B-cell antigen receptor directly regulates BCL-6 stability.

The BCL-6 gene encodes a POZ/Zinc finger protein which functions as a sequence-specific transcriptional repressor expressed in B cells and CD4+ T cells within germinal centers (GC) and downregulated in post-GC B cells. Inactivation of BCL-6 in mice demonstrated that it is required for GC formation, antibody affinity maturation and balanced required for GC formation, antibody affinity maturation and balanced required for GC formation, antibody affinity maturation and balanced Th-2-mediated response. BCL-6 gene expression is commonly deregulated in diffuse large cell lymphoma (DLCL) and, less frequently, in follicular lymphoma (FL) by rearrangements and mutations of its 5′ non-coding region. The mechanism regulating the specific pattern of expression of BCL-6 in GC lymphocytes is unknown. We show that BCL-6 can be phosphorylated by mitogen activated protein kinase(MAPK) in vitro as well as in vivo in 293T cells transfected with vectors expressing BCL-6 and constitutively activated MAPK kinase (MEK-2E). MAPK kinase expression leads to rapid degradation of BCL-6, which does not occur in the presence of inactive MEK or

when BCL-6 phosphorylation mutants are used as targets, indicating that degradation is dependent upon phosphorylation. Phosphorylation-dependent degradation is mediated by the ubiquitin/proteasome pathway since it is prevented by MG132, a specific inhibitor, but not by inhibitors of other proteolytic pathways; in addition, ubiquitin-BCL-6 conjugates can be detected by immunoprecipitation. To demonstrate the physiologic significance of MAPK-mediated phosphorylation/degradation of BCL-6 in B cells, a B cell lymphoma cell line (Ramos) was treated with anti-IgM antibodies with mimics B cell antigen receptor signaling and specifically activates (ERK2) MAPK. This treatment led to rapid BCL-6 phosphorylation followed by ubiquitin/proteasome-dependent degradation. These results identify a MAPK-mediated signaling pathway by which antigen receptor activation causes inactivation of the BCL-6 transcription factor. Since BCL-6 is required for GC formation, this pathway may be critical for the antigen-driven post-GC differentiation of B cells into immunoblasts or memory cells.

Materials and Methods

Reagents and Plasmids

Goat anti-human IgM(μ-heavy chain specific) was obtained from Southern Biotechnology. Polyclonal anti-BCL-6 (N-70-6) antiserum was produced by using the amino-terminal peptides of BCL-6 (Cattoretti et al., 1995). Monoclonal mouse anti-ERK2(C-14) was purchased from Santa Cruz Biotechnology. (Santa Cruz, CA). Monoclonal mouse anti-ubiquitin was obtained from Zymed laboratories, Inc.(South San Francisco, CA). Monoclonal mouse anti-HA (12CA5) was purchased from Boehringer Mannheim, as was Calpain Inhibitor II. Protein A-Sepharose CL-4B and glutathione-Sepharose were purchased from Pharmacia. Myelin basic protein (MBP) and N-CBZ-Leu-Leu-Leu-AL (MG132) were obtained from Sigma. PD098059 was purchased from Calbiochem-Novabiochem (La Jolla, CA). The GST-BCL6,

GST-BCL6ΔZF, GST-BCL6ZF, GST-BCL6ΔZF$_{Ala333}$ and GST-BCL6ΔZF$_{Ala333,343}$ fusion proteins were produced by pGEX-2TK-based plasmids (Pharmacia Biotech) containing full-length, deletion or point mutants of *bcl-6*. The point mutations (*Ala333*, *Ala343*) were generated by PCR-based methods; the sequence of the resulting plasmids was confirmed by nucleotide sequence analysis. pMT2T-BCL-6 and B6BS-TK-LUC have been described as previous (Chang et al., 1996). pMT2T-BCL-6$_{Ala333,343}$ was constructed by transferring the *BclI-Nco*I fragments of plasmid pGEX-2TK-BCL6ΔZF$_{Ala333,343}$ into the pMT2T-BCL-6 vector. MEK-2E-EE-CMV and MEK-EE-CMV for expressing of constitutively active or inactive MEK were provided by D. Templeton (Case Western Reserve University, Cleveland, OH). The pMT2T-HA-BCL-6, pMT2T-HA-BCL-6Δ(300-417), and pMT2T-HA-BCL-6ZF vectors were constructed by inserting the sequences encoding the HA epitope upstream and in frame with *bcl-6* coding sequences. Deletion mutants of *bcl-6* were produced by PCR-based methods and confirmed by sequencing. His$_6$-ubiquitin-CMV was kindly provided by T. Maniatis (Harvard Medical School, Boston, MA). Episomally replicating plasmid, pHeBo-MT which carries EBV oriP, hygromycin B and MT promoter efficiently yields hygromycin-resistant colonies.

ERK2 kinase assays

BCL-6 GST fusion proteins were purified using glutathione-Sepharose beads as suggested by the manufacturer (Pharmacia Biotech). Recombinant ERK2 (New England Biolabs) assays were performed as suggested by the manufacturer using purified wild-type and mutant GST fusion proteins as substrates. In solid-phase ERK2 kinase assays, cells were lysed in ice-cold lysis-buffer (50 mM Tris at pH7.5, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 5 μM $ZnCl_2$, 1 mM Na_3O_4, 10 mM EGTA, 2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml of leupeptin, and 1 μg/ml pepstatin) and centrifuged at 100,000g for 15 min at 4°C. The supernatant (250-500 μg of cellular protein) was then

immunoprecipitated using anti-ERK2 antibodies (C-14) and Protein A-Sepharose CL-4B. Beads were washed three times with lysis buffer and once with kinase buffer (50 mM Tris, pH7.5, 10 mM $MnCl_2$, 5 mM $MgCl_2$). Reactions were initiated by adding 50 μl of kinase buffer containing substrate MBP, 5 μM ATP and 5 μCi [γ-^{32}P] ATP. After 15 min at 37C, reactions were terminated by adding 2x SDS-PAGE sample buffer. Samples were electrophoresed on 15% SDS-polyacrylamide gels which were then dried and analyzed by autoradiography.

Cell transfection

293T cells, grow in DMEM, 10% FBS, were transfected transiently with various DNA vectors using standard calcium phosphate precipitation methods. Ramos cells, grown in IMDM, 10% FBS, were transfected stably with the plasmid pHeBo-MT-HA-BCL-6, pHeBo-MT-HA-BCL-$6_{Ala333,343}$ and the deletion mutant construct pHeBo-MT-HA-BCL-6ZF by electroporation followed by selection in hygromycin B (400 μg/ml). HA-*bcl-6* gene expression under control of the metallothionein (MT) promoter were induced by adding 1 μM of $CdCl_2$.

Northern and Western blot analysis

Total RNA were isolated from cells by using Trizol-reagents (GIBCO-BRL) and equal amounts of RNA were separated on 1% formaldehyde-agarose gel. Northern blot analysis was performed by using standard methods with full-length *bcl-6* cDNA as probes and normalized by GAPDH hybridization. Whole-cell lysates were prepared by lysing cells in RIPA buffer with 2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml of leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, 5 mM NaF, 10 mM β-glycerophosphate. For transient transfectants, protein amounts loaded on gel were normalized by transfection efficiency (β-gal activity). For Ramos cells and their stable transfectants (untreated or treated), equal amounts

of protein were analyzed by 8% or 10% SDS-PAGE, and subsequently by Western blot analysis using anti-BCL-6 (N-70-6), anti-ERK2 (C-14) or anti-HA (12CA5) antibodies at 1:3000, 1:1000 or 1:500 dilutions. The results were visualized by ECL (Amersham).

In vivo ubiquitination assay

293T cells were transfected transiently with pMT2T-BCL-6, His_6-ubiquitin-CMV and MEK-2E-CMV vectors as indicated. MG132 (50 μM) was added 8 hrs after transfection. The total amount of transfected DNA was kept constant in all experiments by adding empty vector. Twenty-four hours after transfection, cells were lysed in RIPA buffer with 10 mM N-ethylmaleimide and various protease inhibitors as described (Pagano et al., 1995). The cell lysates were then immunoprecipitated using anti-BCL-6 antibodies. The immunoprecipitates were loaded on 6% SDS-PAGE and processed for Western blot analysis using the anti-ubiquitin antibodies (Zymed) at 1:1000 dilution as described (Avantaggiati et al., 1996).

Pulse-chase labeling experiment

Ramos cells (12 X 10^7) were collected by centrifugation, washed in PBS, resuspended in 100ml of DMEM without methionine and cysteine (GIBCO-BRL), and starved for 60 min. [^{35}S]methionine and [^{35}S]cysteine (3mCi; ICN) were added and pulse-labeled for 60 min, and then treated with anti-IgM for 30 min. Cold methionine and cysteine were then added to final concentrations of 150 μg/ml. Cells were collected and lysed in RIPA buffer with proteinase and phosphatase inhibitors. The cell extracts, adjusted for equal cpm, were immunoprecipitated with anti-BCL-6 antibodies, and analyzed by SDS-PAGE followed by autoradiography.

<u>Experimental Results</u>

Recent studies have shown that the BCL-6 protein is phosphorylated at multiple sites by mitogen-activated protein kinases (MAPKs), ERK-1 and ERK-2, but not by Jun amino-terminal kinase (JNK) in vitro and in vivo (Moriyama et al., 1997). The results shown in Figure 19 confirm that purified recombinant MAPK (ERK-2) can phosphorylate GST-BCL-6 fusion proteins in vitro. The phosphorylation targets were mapped to the amino-terminal half of the molecule since a carboxy-terminal deletion mutant (GST-BCL-6ΔZF) could be phosphorylated at levels comparable to the wild-type molecule, whereas an amino-terminal deletion mutant (GST-BCL-6ZF) could not be phosphorylated at all (Fig. 19B). Because BCL-6 contains two perfect consensus sites (PXSP) for MAPK-mediated phosphorylation (see Fig. 19A), we generated two mutants (BCL-6_{Ala333} and BCL-$6_{Ala333,343}$) in which one or both of these sites were altered by substituting serines with alanines. These two mutants were phosphorylated at much lower levels than wild type BCL-6, with BCL-$6_{Ala333,343}$ displaying the lowest levels (Fig. 19C). This result indicates that the Ser_{333} and Ser_{343} residues represent a significant fraction, although not all, of the BCL-6 phosphorylation target sites. The residual low level of phosphorylation is consistent with the existence of additional potential MAPK target sequences (SP) clustered within the central domain of the BCL-6 molecule.

MAPK-mediated phosphorylation induces BCL-6 degradation

To determine the effects of MAPK-mediated phosphorylation on *bcl*-6 expression and function, 293T cells (which do not express endogenous BCL-6) were cotransfected with vectors expressing BCL-6, and a MEK (MAP/ERK kinase) mutant (MEK-2E) that functions as a constitutively active MAPK kinase (Yan and Templeton, 1994). Western blot analysis of transfected cell extracts showed that MEK-2E expression (documented by increased ERK2 kinase activity of MEK-2E

transfected cell extracts in solid-phase kinase assays in vitro; Fig. 20A, bottom) induced a dramatic reduction of BCL-6, but not ERK, levels (Fig. 20A, top and middle). The observed reduction in BCL-6 protein levels was dependent upon the phosphorylation activity of MEK-2E, since it did not occur when a vector expressing inactive MEK was cotransfected with *bcl*-6 (Fig. 20B). Northern blot analysis of the same transfected cells showed that the reduction in BCL-6 protein levels were not caused by decreased *bcl*-6 mRNA levels (Fig. 20B, bottom). Furthermore, the MEK-2E-induced decrease in BCL-6 levels was dependent on target phosphorylation, as the partial phosphorylation-resistant mutant $BCL\text{-}6_{Ala333,343}$ was partially resistant to MEK-2E-mediated down-regulation (Fig. 20C). These results indicate that the MEK-2E-induced decrease in BCL-6 levels is not caused by decreased gene transcription or protein synthesis, but rather by decreased protein stability.

Consistent with the MEK-2E-induced reduction in BCL-6 levels, a transient cotransfection assay in 293T cells showed that MEK-2E, but not MEK, could eliminate the transcriptional transrepressor activity of wild-type BCL-6 (Fig. 20D, lanes 3-5) on a reporter vector expressing the luciferase gene downstream to the BCL-6 DNA-binding site (B6BS) (Chang et al., 1996); the partial phosphorylation-resistant mutant $BCL\text{-}6_{Ala333,343}$ was partially resistant to MEK-2E (Fig. 20D, lanes 12-14). Overall, these results indicate that MAPK activation leads to functional inactivation of BCL-6 by causing its accelerated degradation.

BCL-6 degradation is mediated by ubiquitin/proteasome pathway

In examining the possible mechanisms for MAPK-mediated degradation of BCL-6, we noted that the cluster of MAPK putative phosphorylation sites are embedded in a region

enriched in proline, glutamine and serine, within which we identified three typical PEST sequences that score 9.4, 5.0, and 2.6, respectively (Fig. 21A; any score above zero denotes a possible PEST region; scores greater than five indicate the strongest candidates). These motifs have been demonstrated to represent targets for regulated protein degradation (Rogers et al., 1986; Rechsteiner and Rogers, 1996). To determine whether MAPK-mediated BCL-6 degradation targeted these PEST sequences, constructed vectors expressing two epitope HA-tagged *bcl*-6 deletion mutants (see Fig. 21A) and co-transfected them with the MEK-2E vector into 293T cells. Western blot analysis using anti-HA antibodies (Fig. 21B) showed that MEK-2E-mediated degradation targeted the amino-terminal half of the molecule and it was completely abolished in the BCL-6Δ(300-417) internal deletion mutant that lacks a small portion of the BCL-6 protein containing all three PEST sequences. These results indicate that MAPK-induced phosphorylation and degradation of BCL-6 target PEST sequences located in the same domain as the MAPK phosphorylation sites.

The involvement of PEST sequences suggested that MAPK-induced BCL-6 degradation could be mediated by the ubiquitin/proteasome pathway (Hochstrasser, 1996). Therefore, we tested whether MEK-2E mediated degradation of BCL-6 in transfected 293T cells could be inhibited by the proteasome inhibitor Cbz-LLL (MG132) (Kim and Maniatis, 1996; Palombella et al., 1994; Rock et al., 1994). Figure 22A shows that BCL-6 degradation was completely inhibited by MG132, but not by DMSO (solvent control) or calpain inhibitor II (CI II), a cysteine-protease inhibitor (Kim and Maniatis, 1996; Palombella et al., 1994). Because the addition of multiple ubiquitins to the proteolysis substrate is a key step preceding target degradation by the proteasome, we then tested whether BCL-6/ubiquitin conjugates in vivo could be detected. To this end, 293T cells were transfected with vectors expressing BCL-6,

MEK-2E and epitope (His_6)-tagged ubiquitin in the presence or absence of MG132. Cell lysates were subjected to immunoprecipitation with anti-BCL-6 antibodies, and the immunoprecipitates were analyzed by Western blotting using anti-ubiquitin antibodies. Fig. 22B shows that in the absence of MG132, low levels of BCL-6/ubiquitin were detectable when BCL-6 and ubiquitin were co-expressed with exogenous MEK-2E (lane 4); in the presence of MG132, typical ladders representing multi-ubiquitinated forms of BCL-6 were detectable at high levels in the presence of MEK-2E (lane 8); low levels were detectable also in its absence (lane 7), suggesting that the normal turn-over of BCL-6 degradation may be mediated by basal levels of endogenous MAPK activity. Based on the specific pharmacological inhibition and the detection of MEK-2E-inducible BCL-6/ubiquitin conjugates, we conclude that MAPK-induced phosphorylation induces degradation of BCL-6 via the ubiquitin/proteasome pathway.

MAPK-mediated phosphorylation and degradation of BCL-6 is induced by antigen-receptor signaling in B Cells

To demonstrate the physiological significance of MAPK-mediated phosphorylation/degradation of BCL-6 in B cells, we treated a B-cell lymphoma cell line (Ramos) with anti-IgM antibodies, a treatment that mimics B-cell antigen-receptor signaling and specifically activates MAPK (ERK2) (Gold et al., 1992; Sakata et al., 1995; Sutherland et al., 1996). As previously demonstrated, an in vitro assay showed that ERK2 kinase activity was rapidly increased 5 min after anti-IgM treatment (Fig. 23A); this was followed by hyperphosphorylation of ERK2 and BCL-6 (note the slow migrating bands in Fig. 23A) and by the disappearance of BCL-6, but not ERK2. In the same experiment, Northern blot analysis showed that *bcl*-6 mRNA levels did not change during anti-IgM treatment of Ramos cells (Fig. 23A, bottom). In order to determine whether hyperphosphorylation was associated with increased BCL-6

instability, we analyzed the half-life of BCL-6 in anti-IgM-treated Ramos cells by a "pulse-chase" labeling experiment. The results (Fig. 23B) showed that the hyperphosphorylated (slow-migrating) forms of BCL-6 were significantly less stable than the hypophosphorylated (fast-migrating) forms (half life 4-6 hrs.). Anti-IgM-induced BCL-6 degradation was dependent upon phosphorylation since it was inhibited by a specific MAPK inhibitor PD098059 (Fig. 23C) (Dudley et al., 1995; Pang et al., 1995), and was mediated by the ubiquitin/proteasome pathway since was specifically inhibited by MG132 (Fig. 23D). Finally, anti-IgM treatment of Ramos cells stably transfected with Cadmium-inducible vectors expressing HA-tagged wild-type, 333/343 mutant, or amino-terminal deleted BCL-6 proteins showed that degradation required phosphorylation of the 333 and 343 serines as well as the amino-terminal half of BCL-6 containing the PEST motifs (Fig. 23E). These results demonstrate that MAPK-mediated phosphorylation of BCL-6 and its degradation by the ubiquitin/proteasome pathway represent a physiologic pathway that can be activated by antigen receptor signaling in B cells.

<u>Experimental Discussion</u>

The present study identifies a signal transduction pathway by which the antigen receptor regulates the stability of the BCL-6 transcription factor in B cells. The results have implications for the normal mechanism regulating GC formation as well as for the role of deregulated *bcl*-6 expression in lymphomas deriving from GC B cells. In addition, several observations suggest that MAPK-mediated regulation of POZ/Zinc finger protein stability may represent a general, highly conserved regulatory mechanism in eukaryotic cells.

Regulation of BCL-6 stability during GC formation

The finding that antigen-receptor-induced activation of MAPK leads to BCL-6 degradation must be seen in the context of the complex network of signals modulating receptor signaling in GC B cells (Tedder, et al., 1997; Cambier, 1997). During GC formation, activation of this pathway is consistent with the observation that pre-GC B cells in the follicular mantle zone, the site where B cells encounter the antigen, express *bcl*-6 RNA, but not the BCL-6 protein (Allman et al., 1996; Cattoretti et al., 1995). Within the GC, the coexistence of antigen and *bcl*-6 expression implies that antigen-receptor signaling must be modulated by mechanisms that allow BCL-6 stability. These mechanisms may include down-regulation of antigen-receptor expression in centroblasts (MacLennan, 1994), modulation of receptor signaling by CD22 or Fc γ receptor (Tedder, et al., 1997; Cambier, 1997), and the activity of de-ubiquitinases (DUB), which regulate substrate ubiquitination and are induced by cytokines acting on GC B cells (Zhu et al., 1996). During post-GC differentiation, antigen-induced degradation may serve as a rapid mechanism to down-regulate *bcl*-6 expression, in synergy with transcriptional down-regulation by CD40 signaling (Allman et al., 1996; Cattoretti et al., 1997). Finally, the regulation of BCL-6 stability during GC development is likely to be affected by various additional signals that activate MAPK in B cells including various cytokines (TNF, IL-6, IL-2) (Vietor et al., 1993; Minami et al., 1994; Fukada et al., 1996). The effect of these signals on the pathway linking the antigen receptor to BCL-6 can be tested in the experimental systems used in this study.

Implication for lymphomagenesis

Most B-cell lymphoma types, including follicular (FL), diffuse large cell, (DLCL) and Burkitt (BL) lymphoma, are thought to derive from the GC B cells. Although rearrangements and/or mutations of the *bcl*-6 regulatory region are found most frequently in DLCL and FL, all

GC-derived lymphoma, including those carrying a structurally normal *bcl*-6 gene, express the BCL-6 protein (Cattoretti et al., 1995). This implies that the BCL-6 protein is stable in tumor cells and suggests that MAPK-mediated degradation may be blocked by genetic or epigenetic alterations affecting the pathway leading to BCL-6 degradation. The observation that BCL-6 degradation can be triggered from the cell surface by activation of the antigen receptor has potential relevance for the therapy of B-cell lymphoma.

MAPK-mediated regulation of POZ/Zinc finger transcription factors

MAPK is a ubiquitous, evolutionarily conserved signal transducer that is activated by heterogeneous signals that originate from the cell membrane and are transduced to MAPK via RAS proteins (Gold and Matsuuchi, 1995; Alberola-Ila et al., 1997). Accordingly, POZ/zinc finger proteins represent a large family of highly conserved transcription factors including *Drosophila* cell fate regulators such as *Tramtrack* and *Broad-complex*, as well as human cancer-associated proteins such as BCL-6 and PLZF. These molecules have strong structural (POZ and ZF domains), as well as functional homologies being transcriptional repressors that control cell differentiation (Albagli et al., 1995; Chen et al., 1994; Emery et al., 1994). Most notably, POZ/zinc finger proteins also carry possible MAPK phosphorylation sites and PEST sequences in approximately the same position as those carried by BCL-6 (Niu et al., unpublished observation). In *Drosophila*, degradation of TTK88, a POZ/zinc finger inhibitor of neural-cell differentiation, has been shown to be mediated by MAPK (Li et al., 1997; Tang et al., 1997). Thus, degradation of POZ/zinc finger transcription factors may represent a general mechanism by which the RAS/MAPK pathway controls cell function and differentiation.

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What is claimed is:

1. A method of degrading BCL-6 in cells comprising: administering a molecule which induces phosphorylation of BCL-6 and thereby induces BCL-6 degradation.

2. The method of claim 1 wherein the molecule which induces phosphorylation of the BCL-6 is a mitogen-activated protein kinase (MAPK).

3. The method of claim 1 wherein the molecule which induces phosphorylation of the BCL-6 is a functionally active mutant of a mitogen-activated protein kinase (MAPK).

4. The method of claim 2 wherein the MAPK is ERK-1 or ERK-2.

5. The method of claim 1, wherein the BCL-6 is phosphorylated either at one site or at multiple sites.

6. The method of claim 1, wherein the molecule which induces phosphorylation of the BCL-6 is a molecule which activates an antigen receptor on B cell surfaces.

7. The method of claim 6, wherein the molecule which activates an antigen receptor on B cell surfaces is an antibody.

8. The method of claim 7, wherein the antibody is an anti-IgM antibody.

9. The method of claim 6, wherein the molecule which activates an antigen receptor on B cell surfaces is a molecule which activates MAPK in B cells.

10. The method of claim 9, wherein the molecule which activates MAPK in B cells is a cytokine.

11. The method of claim 10, wherein the cytokine is TNF, IL-6, or IL-2.

12. The method of claim 1, wherein the molecule is cross-linked to a B cell antigen receptor to activate the receptor.

13. The method of claim 1, wherein cross-linking the molecule to the B cell antigen receptor activates the MAPK.

14. A method of treating a subject with lymphoma which comprises:

    administering an effective amount of a pharmaceutical composition comprising a molecule which induces phosphorylation of BCL-6 protein so as to induce degradation of BCL-6 and a pharmaceutically acceptable carrier, thereby treating the subject with lymphoma.

15. The method of claim 14, wherein the lymphoma expresses BCL-6.

16. The method of claim 14, wherein the pharmaceutical composition comprises a MAPK activator.

17. The method of claim 16, wherein the MAPK activator is an antibody.

18. The method of claim 17, wherein the antibody is an anti-IgM antibody.

19. The method of claim 16, wherein the MAPK activator is a cytokine.

20. The method of claim 19, wherein the cytokine is TNF, IL-6, or IL-2.

21. The method of claim 14, wherein the lymphoma is a B-cell lymphoma.

22. The method of claim 21, wherein the B-cell lymphoma is derived from germinal center B cells.

23. The method of claim 14, wherein the administration of the pharmaceutical composition is intravenous or intratumor.

24. A method of regulating deceasing BCL-6 levels in cells comprising administering a compound which interferes with transcription of bcl-6 and thereby prevents expression of BCL-6 protein so as to thereby deceasing BCL-6 levels in the cells.

25. The method of claim 24, wherein the compound which interferes with transcription of bcl-6 prevents binding of a transcription factor and histone acetylase/deacetylase complexes.

26. The method of claim 25, wherein the compound is N,N'-hexamethylene bisacetamide (HMBA) or trichostatin.

27. A method of treating lymphoma comprising decreasing BCL-6 levels in cells comprising the method of claim 24.

FIGURE 1

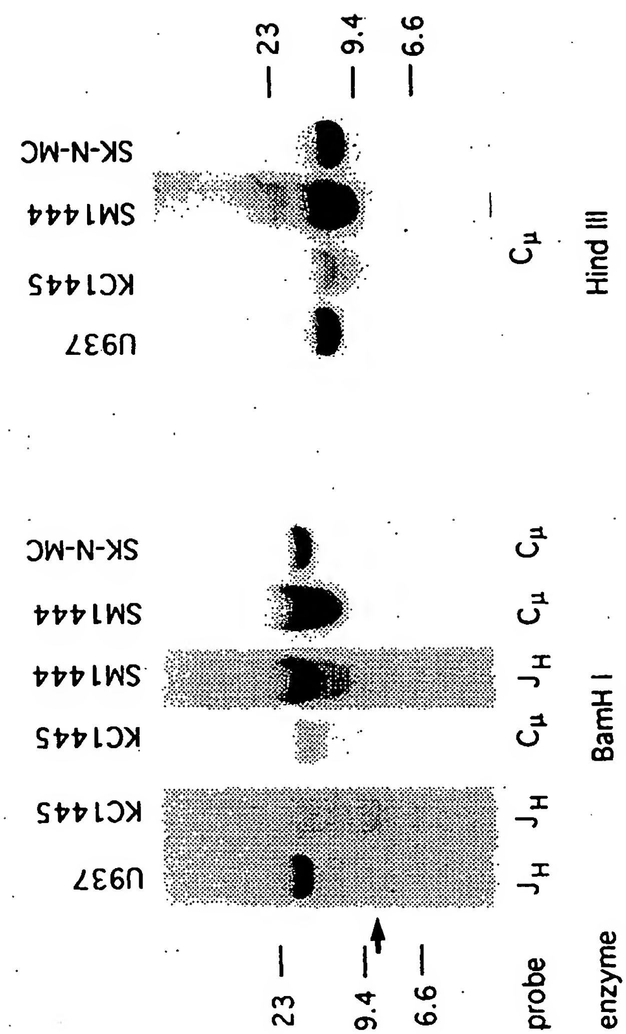

U937
KC1445
KC1445
SM1444
SM1444
SK-N-MC
23
9.4
6.6
probe
enzyme
J_H
J_H
C_μ
J_H
C_μ
C_μ
BamH I
U937
KC1445
SM1444
SK-N-MC
C_μ
Hind III

FIGURE 2

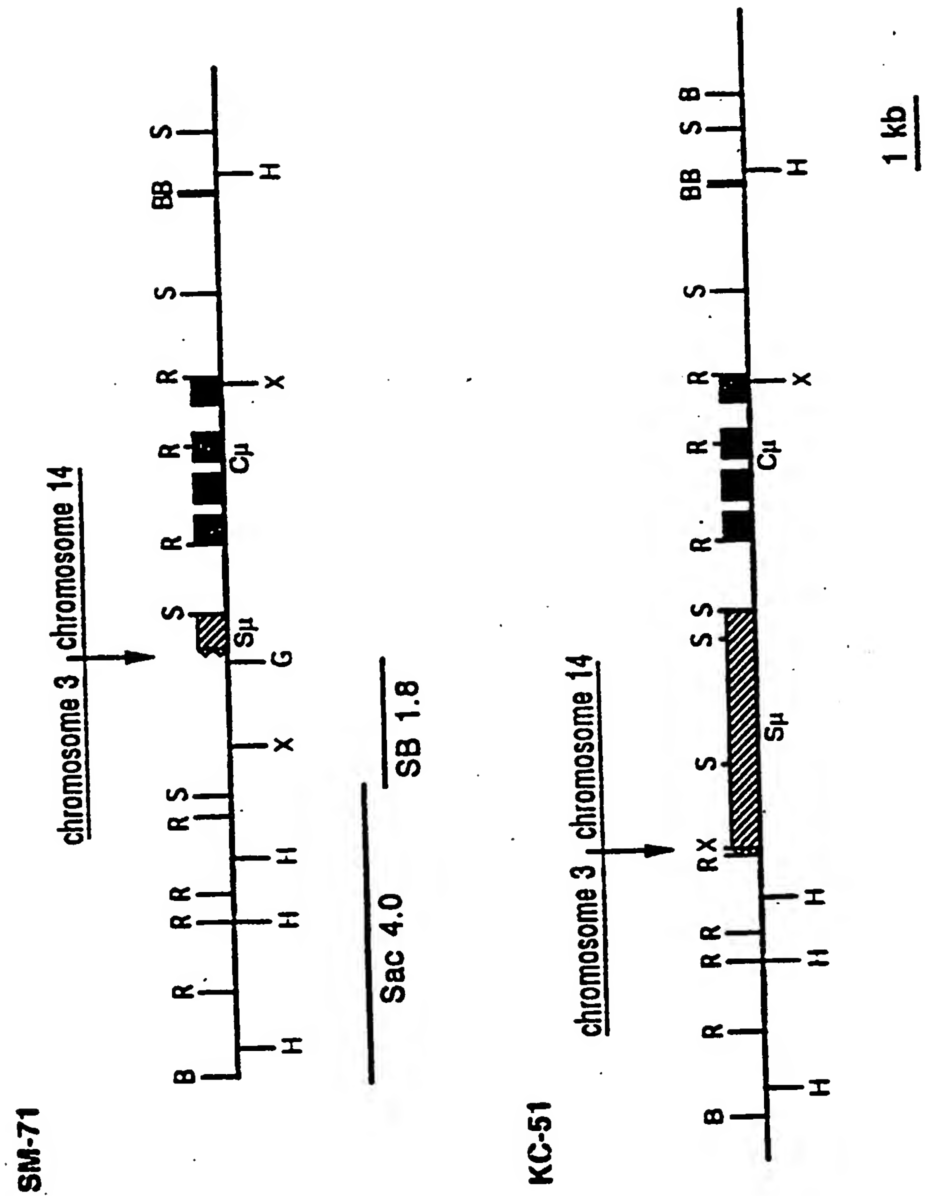

SM-71
chromosome 3
chromosome 14
Sμ
Cμ
Sac 4.0
SB 1.8
KC-51
chromosome 3
chromosome 14
Sμ
Cμ
1 kb

FIGURE 3A FIGURE 3B

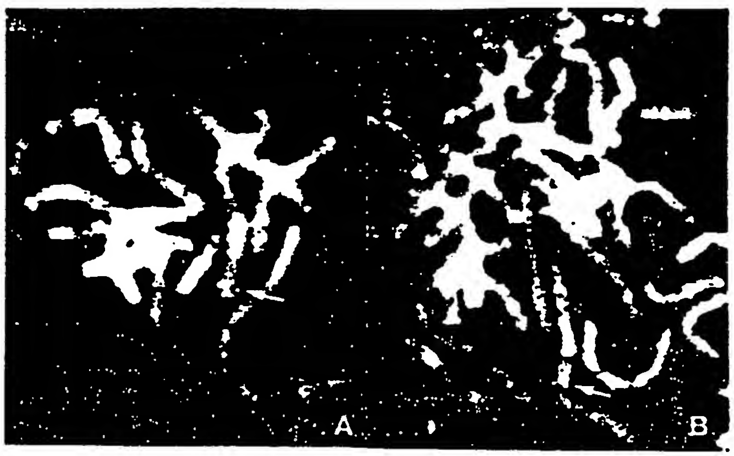


FIGURE 4C

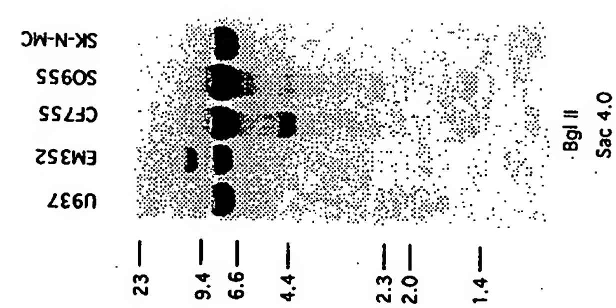


FIGURE 4B

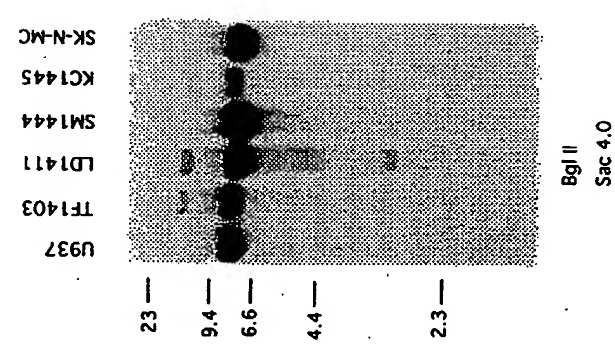


FIGURE 4A

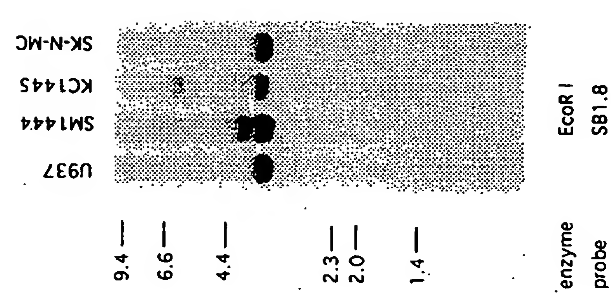
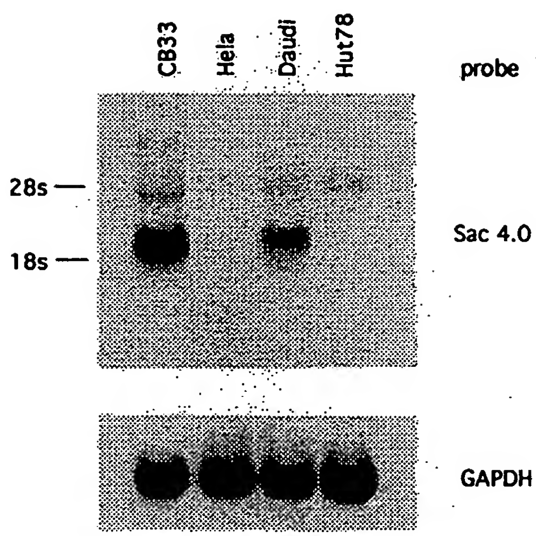


FIG. 5



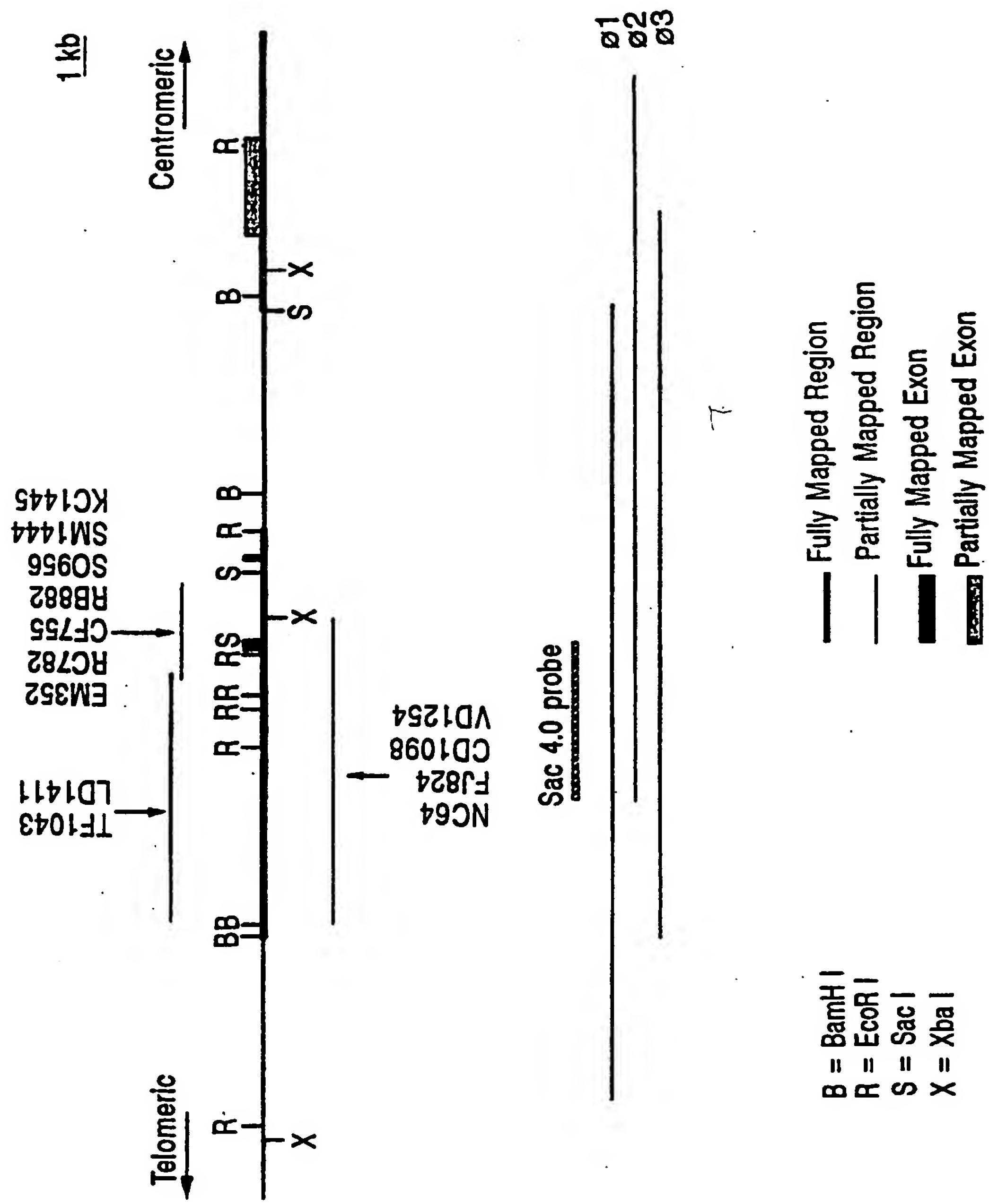
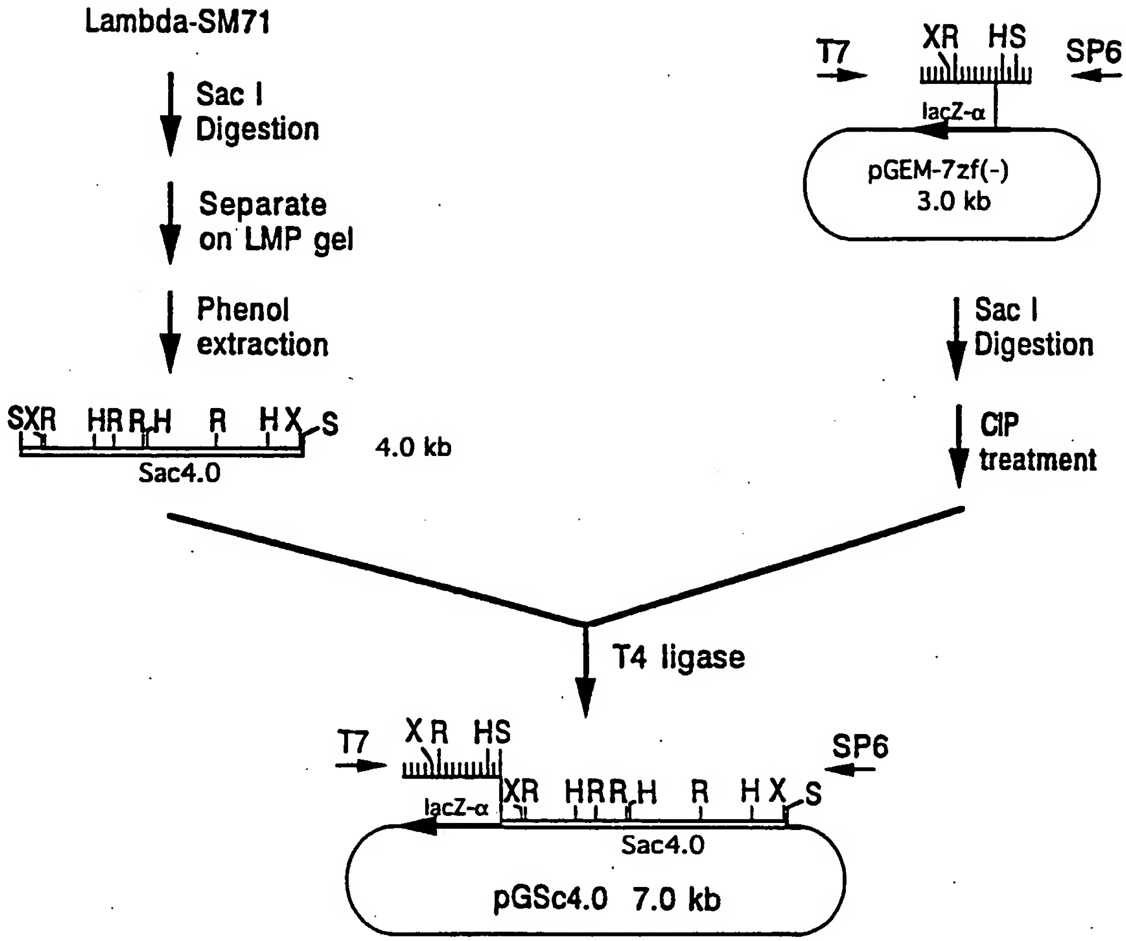


FIG. 6

Map of Human *BCL-6* Locus

FIG. 7



Lambda-SM71 = a recombinant Lambda phage clone containing *Bcl-6* breakpoint

H = Hind III
R = EcoR I
S = Sac I
X = Xho I

FIG. 8

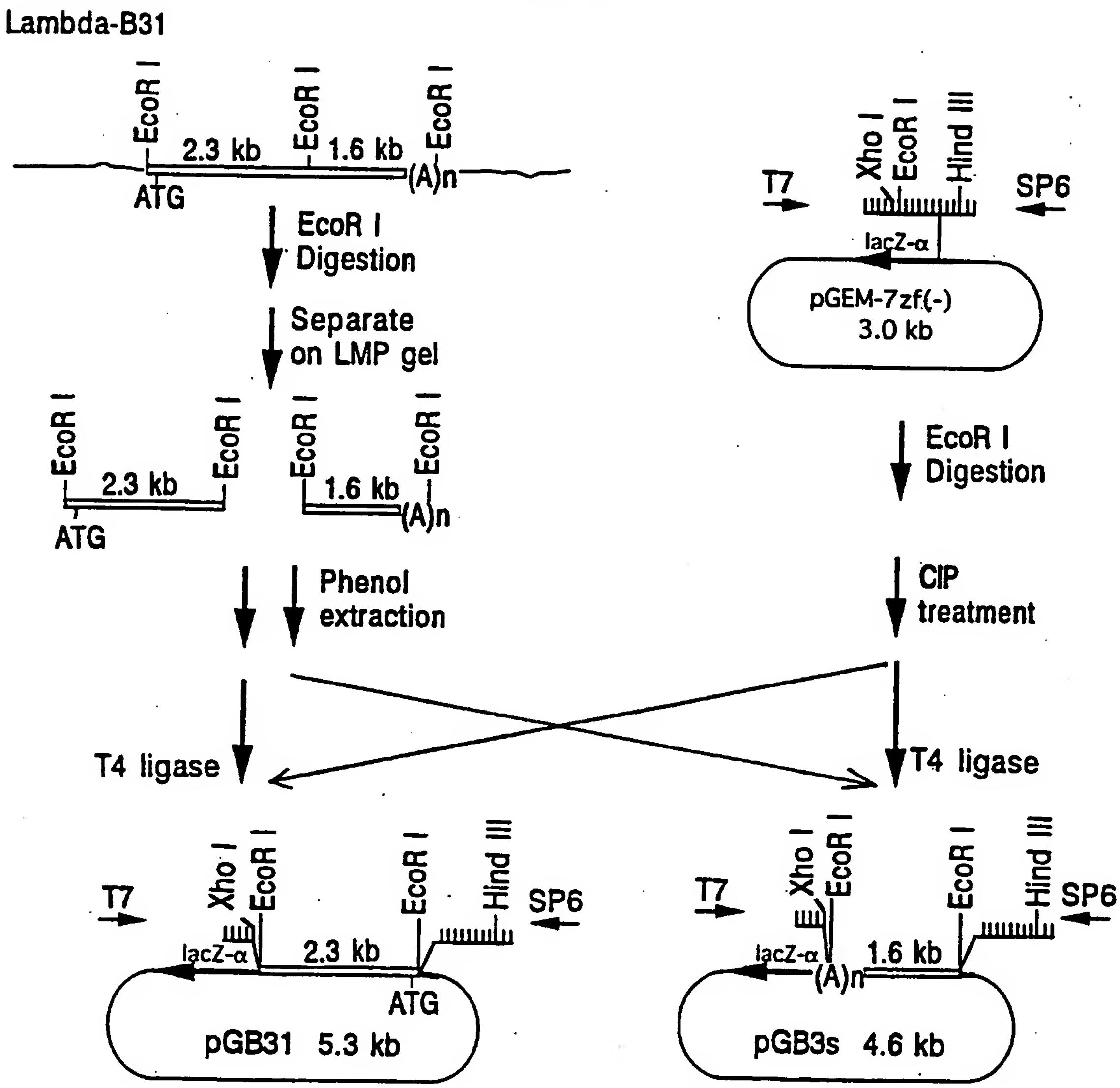

Lambda-B31
EcoR I
EcoR I
EcoR I
2.3 kb
1.6 kb
ATG
(A)n
EcoR I
Digestion
Separate
on LMP gel
Phenol
extraction
T4 ligase
T7
Xho I
EcoR I
Hind III
SP6
lacZ-α
pGEM-7zf(-)
3.0 kb
EcoR I
Digestion
CIP
treatment
T4 ligase
pGB31 5.3 kb
pGB3s 4.6 kb

FIGURE 9A

SEQ. ID. NOS. 1-2

cDNA and Amino Acid Sequences of BCL-6

```
1   [illegible]                                                  120
121 [illegible]                                                  240
241 [illegible]                                                  360
                            M A S P A D S C I Q P                 11

361 [illegible]                                                  480
12  T R H A R D V L L N L N R L R S R D I L T D V V I V V S R E Q F R A H K T V L M   51

481 [illegible]                                                  600
52  A W R G L F Y S I F T D Q L K C N L S V I N L D P E I N P E G F C I L L D F M Y   91

601 [illegible]                                                  720
92  T S R L N L R E G N I M A V M A T A M Y L Q M E H V V D T C R K F I K A S E A E   131

721 [illegible]                                                  840
132 M V S A I K P P R E E F L N S R M L M P Q D I M A Y R G R E V V E N N L P L R S   171
```

FIGURE 9B

841 [illegible] 960
172 A P G C E S R A F A P S L Y S G L S T P P A S Y S M Y S H L P V S S L L F S D E 211

961 [illegible] 1080
212 E F R D V R M P V A N P F P K E R A L P C D S A R P V P G E Y S R P T L E V S P 251

1081 [illegible] 1200
252 N V C H S N I Y S P K E T I P E R A R S D M H Y S V A E G L K P A A P S A R N A 291

1201 [illegible] 1320
292 P Y F P C D K A S K E E E A P S S E D E I A L H F E P P N A P L N R K G L V S P 331

1321 [illegible] 1440
332 Q S P Q K S D C Q P N S P T E A C S S K N A C I L Q G S G S P P A K S P T D P K 371

1441 [illegible] 1560
372 A C S W K K Y K F I V L N S L N Q N A K P G G P E Q A E L G [illegible] L S P R A Y T A P 411

FIGURE 9C

```
1561 CCTGCCTGCCAGCCACCCCATGGAGCCTGAGAACCTTGACCTCCAGTCCCCAACCAAGCTGAGTGCCAGCGGGGAGGACTCCACCATCCCACAAGCCAGCCGGCTCAATAACATCGTTAAC 1680
412  P  A  C  Q  P  P  M  E  P  E  N  L  D  L  Q  S  P  T  K  L  S  A  S  G  E  D  S  T  I  P  Q  A  S  R  L  N  N  I  V  N  451

1681 AGGTCCATGACGGGCTCTCCCCGCAGCAGCAGCGAGAGCCACTCACCACTCTACATGCACCCCCCGAAGTGCACGTCCTGCGGCTCTCAGTCCCCACAGCATGCAGAGATGTGCCTCCAC 1800
452  R  S  M  T  G  S  P  R  S  S  S  E  S  H  S  P  L  Y  M  H  P  P  K  C  T  S  C  G  S  Q  S  P  Q  H  A  E  M  C  L  H  491

1801 ACCGCTGGCCCCACGTTCGCTGAGGAGATGGGCGAGACCCAGTCTGAGTACTCAGATTCTAGCTGTGAGAACGGGGCCTTCTTCTGCAATGAGTGTGACTGCCGCTTCTCTGAGGAGGCC 1920
492  T  A  G  P  T  F  A  E  E  M  G  E  T  Q  S  E  Y  S  D  S  S  C  E  N  G  A  F  F  C  N  E  C  D  C  R  F  S  E  E  A  531

1921 TCACTCAAGAGGCACACGCTGCAGACCCACAGTGACAAACCCTACAAGTGTGACCGCTGCCAGGCCTCCTTCCGCTACAAGGGCAACCTCGCCAGCCACAAGACCGTCCATACCGGTGAG 2040
532  S  L  K  R  H  T  L  Q  T  H  S  D  K  P  Y  K  C  D  R  C  Q  A  S  F  R  Y  K  G  N  L  A  S  H  K  T  V  H  T  G  E  571

2041 AAACCCTATCGTTGCAACATCTGTGGGGCCCAGTTCAACCGGCCAGCCAACCTGAAAACCCACACTCGAATTCACTCTGGAGAGAAGCCCTACAAATGCGAAACCTGCGGAGCCAGATTT 2160
572  K  P  Y  R  C  N  I  C  G  A  Q  F  N  R  P  A  N  L  K  T  H  T  R  I  H  S  G  E  K  P  Y  K  C  E  T  C  G  A  R  F  611
```

FIGURE 9D

2161 GTACAGGTGGCCGAGCTCCGTGCCGAGGTGCTTATCCACACTGGTGAGAAGCCCTATCCCTGTGAAATCTGTGGCACCCGGTTTCGCCACCTTCAGACTCTGAAGAGCCACCTGCGAATC 2280
612 V Q V A E L R A E V L I H T G E K P Y P C E I C G T R F R H L Q T L K S H L R I 651

2281 CACACAGGAGAGAAACCTTACCATTGTGAGAAGTGTAACCTGCATTTCCGTCACAAAAGCCAGCTGCGACTTCACTTGCGCCAGAAGCATGGCGCCATCACCAACACCAAGGTGCAATAC 2400
652 H T G E K P Y H C E K C N L H F R H K S Q L R L H L R Q K H G A I T N T K V Q Y 691

2401 CGGGTGTCAGCCACTGACCTGCCTCCGGAGCTCCCCAAAGCCTGCTGAAGCCATGGAGTGTTGATGCTTTCGTCTCCAGCCCCTTCTCA AATCTACCCAAAGGATACTGTAACACTTTAC 2520
692 R V S A T D L P P E L P K A C *

2521 AATGTTCATCCCATGATGTAGTGCCTCTTTCATCCACTAGTGCAAATCATAGCTGGGGGTTGGGGGTGGTGGGGGTCGGGGGCCTGGGGGACTGGGAGCCCCAGCAGCTCCCCCTCCCCCA 2640

2641 CTGCCATAAAACATTAAGAAAATCATATTGCTTCTTCTCCTATGTG .... (~560 bp) .... TTTAAGTATTGCAT

CTGTATAAGTAAGAAAATATTTTTGTCTAAAATGCCTCAGTGTATTTGTATTTTTTTGCAAGTGGGGGGTTACAATTTACCCAGTGTGTATTAAAAAAAAACCCAAAGAACCCAA AATCTC

CAGAAGGAAAAATGTGTGATTTTTGTTCTAGTTTTCAGTTTGTATGTATCCCGTACAACTGTGTCCTCAGGGTGCCTTTTTTCACCGAAGTTTTCAATGATGGGGGAGGGTGCACCCATCCCTT

TTTGAAGTGTACGCAGACACAGGGACTTGAAGTTGTTACTAACTAAACTCTCTTTGGGAATGTTTGTCTCATCCCANTCTGGGTCATGCTTGTGTGATAACTACTCCCGAGACACGGTTT

GGCTGTGTCTAAACTGCATTACCCCGTTGTAAAAAATAGCTGTACCAATATAAGAATAAAATGTTGGAAAGTGGCAAAAAAAAAAAAAAAAAAAAAAAAA

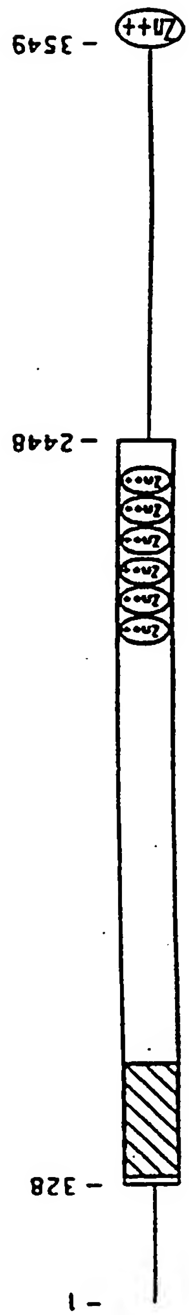


FIGURE 10A

FIGURE 10B

```
1   MASPADSCIQ FTRHASDVLL NLNRLRSRDI LTDVVIVVSR EQFRAHKTVL
51  MACSGLFYSI FTDQLKCNLS VINLDPEINP EGFCILLDFM YTSRLNLREG
101 NIMAVMATAM YLQMEHVVDT CRKFIKASEA EMVSAIKPPR EEFLNSRMLM
151 PQDIMAYRGR EVVENNLPLR SAPGCESRAF APSLYSGLST PPASYSMYSH
201 LPVSSLLFSD EEFRDVRMPV ANPFPKERAL PCDSARPVPG EYSRPTLEVS
251 PNVCHSNIYS PKETIPEEAR SDMHYSVAEG LKPAAPSARN APYFPCDKAS
301 KEEERPSSED EIALHFEPPN APLNRKGLVS PQSPQKSDCQ PNSPTEACSS
351 KNACILQASG SPPAKSPTDP KACNWKKYKF IVLNSLNQNA KPGGPEQAEL
401 GRLSPRAYTA PPACQPPMEP ENLDLQSPTK LSASGEDSTI PQASRLNNIV
451 NRSMTGSPRS SSESHSPLYM HPPKCTSCGS QSPQHAEMCL HTAGPTFAEE
501 MGETQSEYSD SSCENGAFFC NECDCRFSEE ASLKRHTLQT HSDKPYKCDR
551 CQASFRYKGN LASHKTVHTG EKPYRCNICG AQFNRPANLK THTRIHSGEK
601 PYKCETCGAR FVQVAHLRAH VLIHTGEKPY PCEICGTRFR HLQTLKSHLR
651 IHTGEKPYHC EKCNLHFRHK SQLRLHLRQK HGAITNTKVQ YRVSATDLPP
701 ELPKAC*
```

FIG. 11

ZFPJS	(2-56)	DGSFVQHSVRVLQELNKQREKGQYCDATLDVGGLVFKAHWSVLACCSHFFQSLYG
KUP	(1-54)	.MDTASHSLVLLQQLNMQREFGFLCDCTVAIGDVYFKAHRAVLAAFSNYFKMIFI
VA55R	(1-51)	....MNNSSELIAVINGFRNSGRFCDISIVINDERINAHKLILSGASEYFSILFS
ttk	(9-63)	CLRWNNHQSNLLSVFDQLLHAETFTDVTLAVEGQHLKAHKNVLSACSPYFNTLFV
kelch	(132-186)	QYSNEQHTARSFDAMNEMRKQKQLCDVILVADDVEIHAHRMVLASCSPYFYAMFT
PLZF	(10-63)	QLQNPSHPTGLLCKANQMRLAGTLCDVVIMVDSQEFHAHRTVLACTSKMFEILF.
BCL-6	(8-62)	CLQFTRHASDVLLNLNRLRSRDILTDVVIVVSREQFRAHKTVLMACSGLFYSIFT
ZFPJS	(57-104)	DG..SGGSV.VLPAGF.AEIFGLLLDFFYTGHLALTSGNRDQVLLAARELRV...
KUP	(55-107)	HQ..ISECIKIQPTDIQPDIFSYLLHIMYTGKGPKQIVDHSRLEEGIRFLHADYL
VA55R	(52-106)	NNFIDSNEYEVNLSHLDYQSVNDLIDYIYGIPLSLTNDNVKYILSTADFLQIGSA
ttk	(64-116)	SH..PEKHPIVILKDVPYSDMKSLLDFMYRGEVSVDQERLTAFLRVAESLRIKGL
kelch	(187-240)	SFEESRQARITLQS.VDARALELLIDYVYTATVEVNEDNVQVLLTAANLLQLTDV
PLZF	(64-114)	...HRNSQHYTLDF.LSPKTFQQILEYAYTATLQAKAEDLDDLLYAAEILEIEYL
BCL-6	(63-117)	DQLKCNLSVINLDPEINPEGFCILLDFMYTSRLNLREGNIMAVMATAMYLQMEHV

FIGURE 12

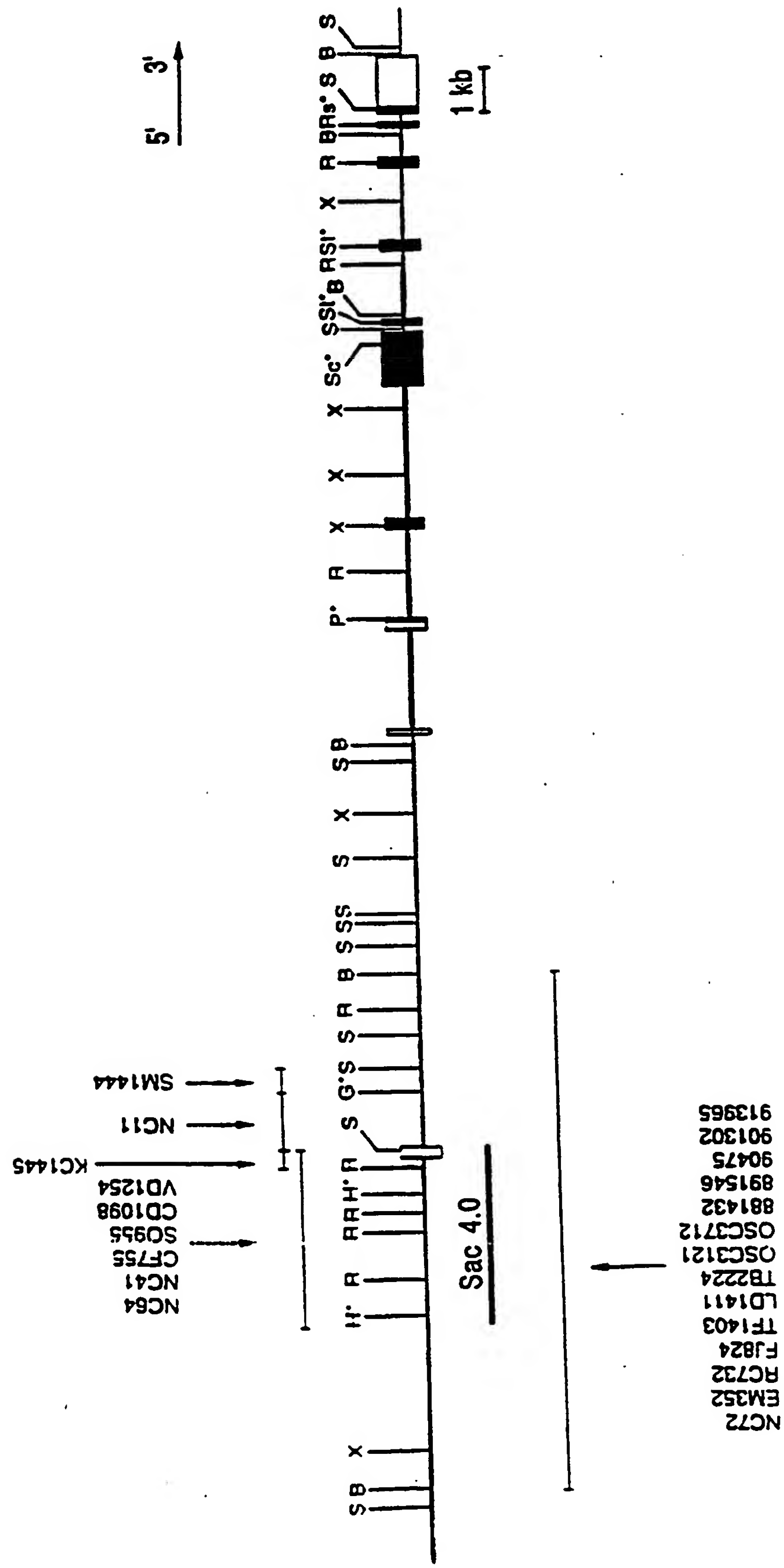
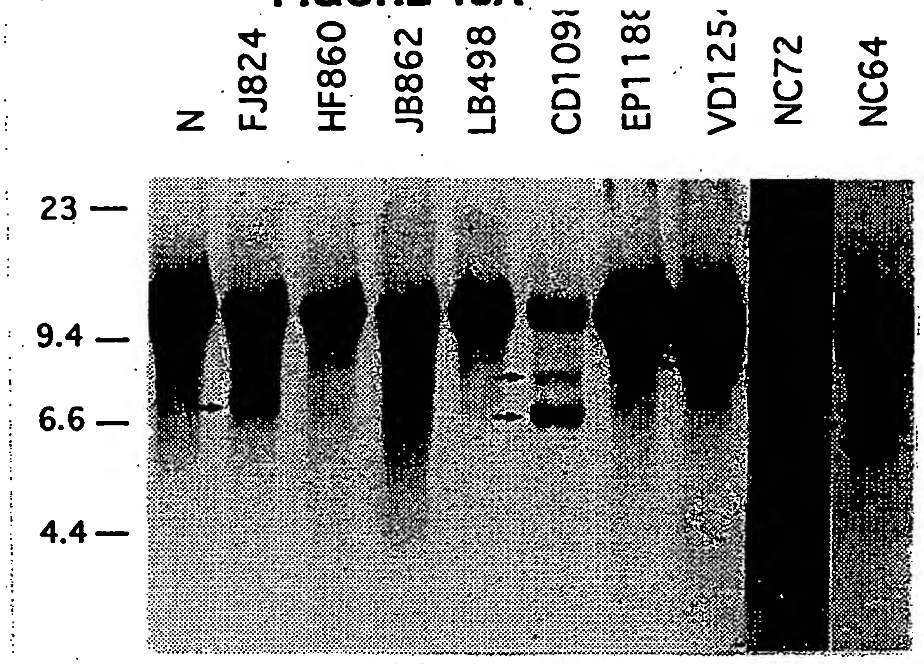
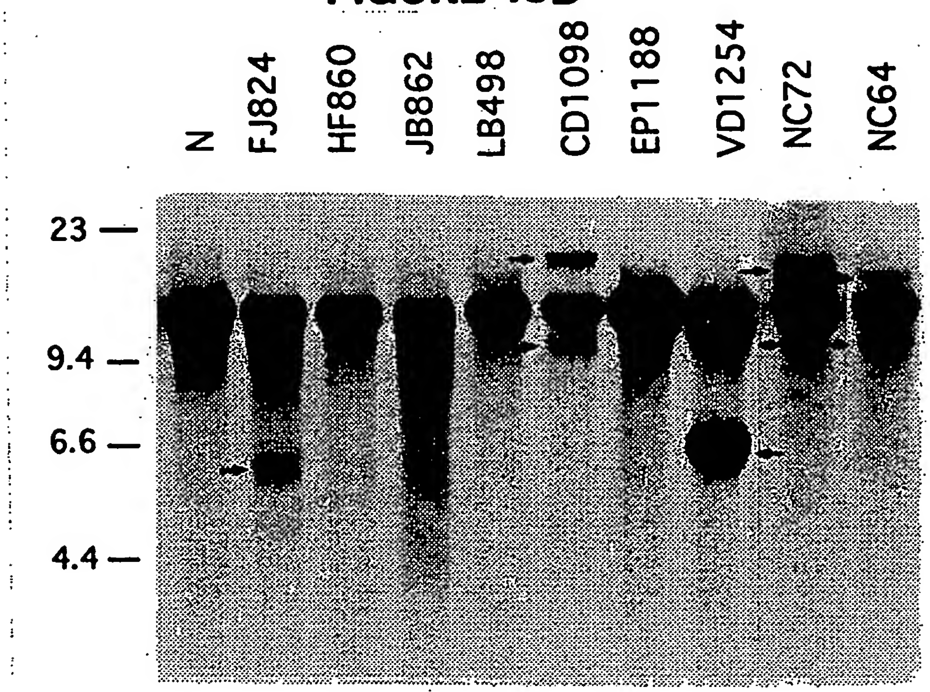

5'
3'
1 kb
Sac 4.0
SM1444
NC11
KC1445
VD1254
CD1098
SO955
CF755
NC41
NC64
913965
901302
90475
891546
881432
OSC3712
OSC3121
TB2224
LD1411
TF1403
FJ824
RC732
EM352
NC72

FIGURE 13A



BamH I

FIGURE 13B



Xba I

FIGURE 14A

FIGURE 14B

FIGURE 14C

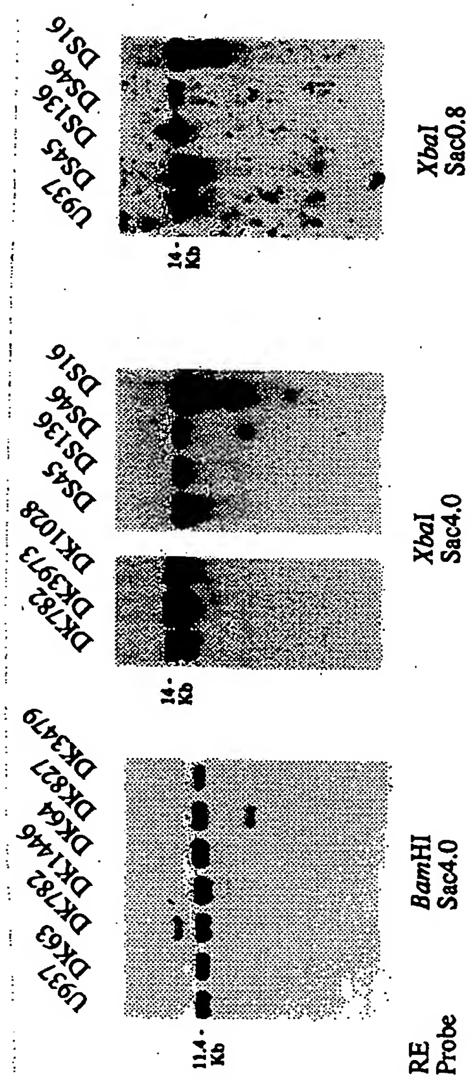
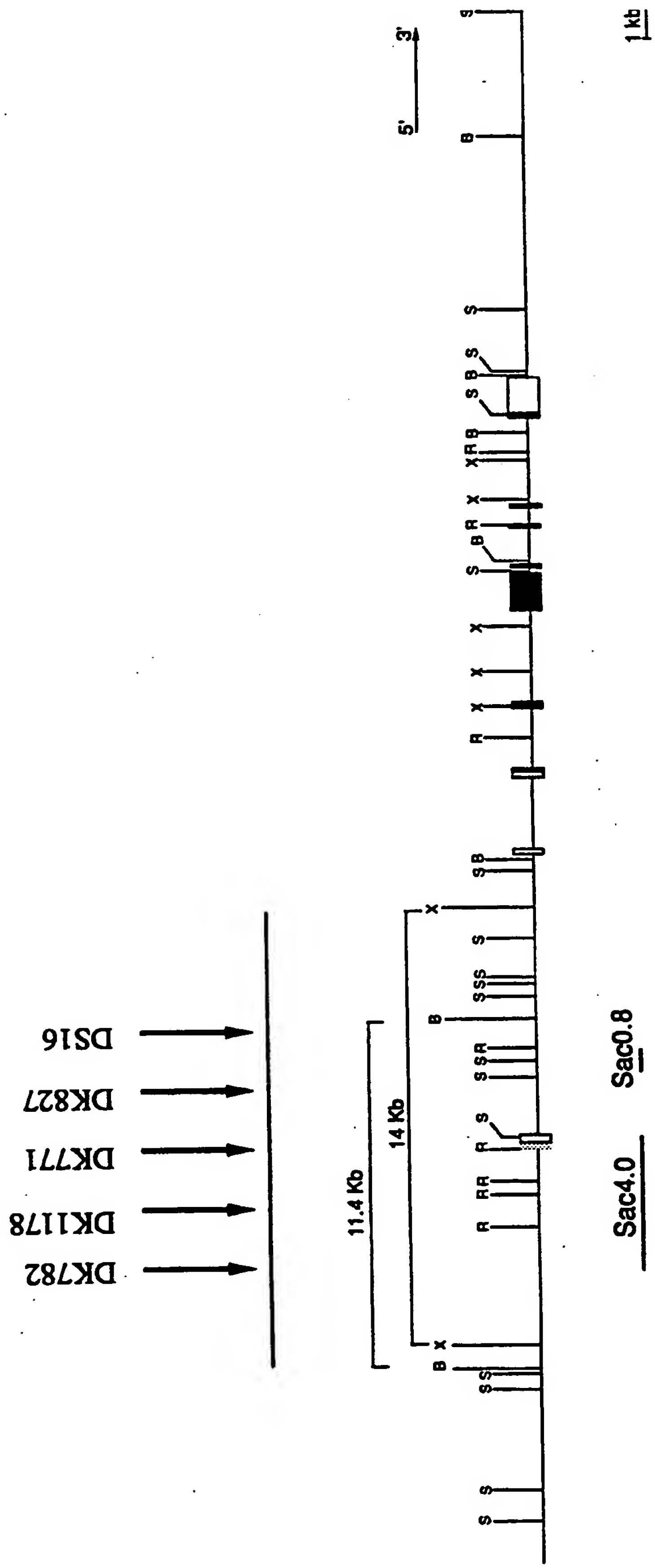


FIGURE 15



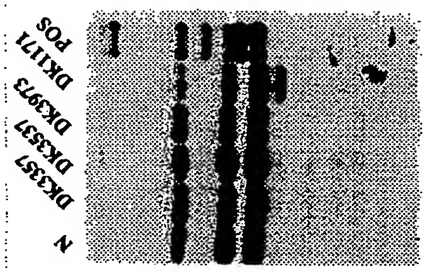


FIGURE 16C

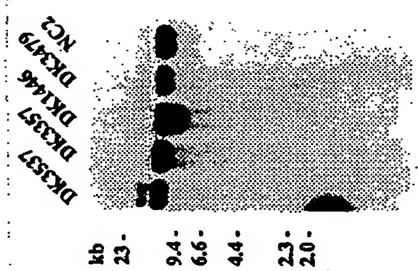


FIGURE 16B

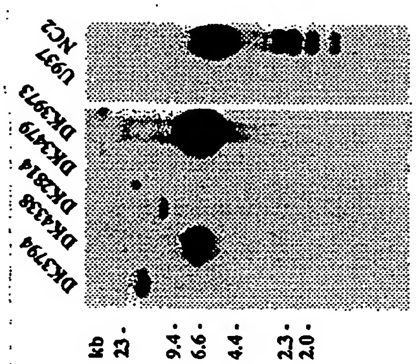
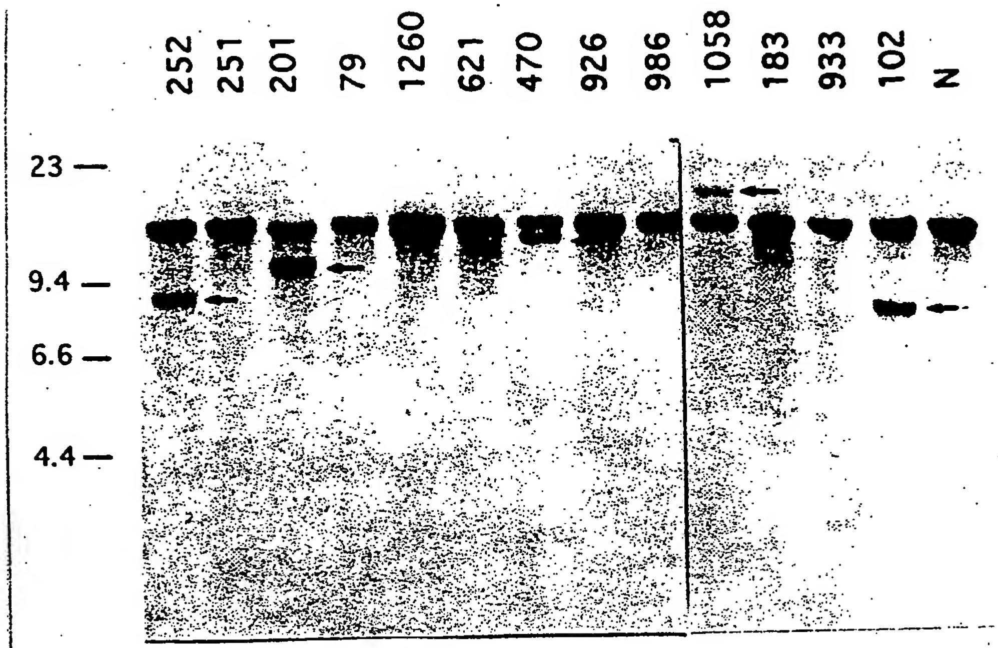


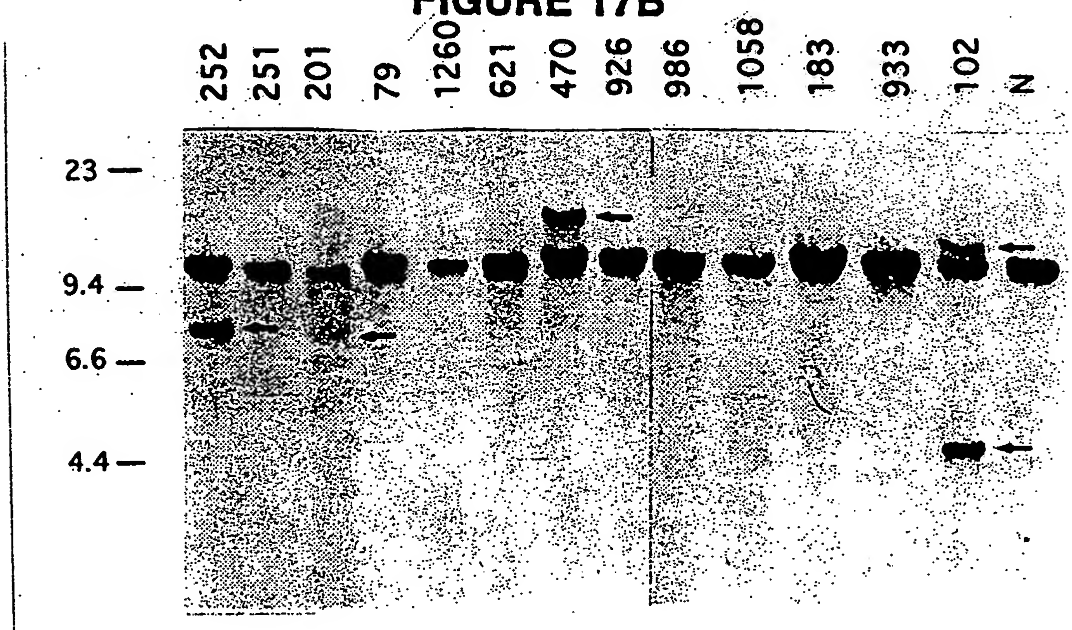
FIGURE 16A

FIGURE 17A



Xba I

FIGURE 17B



BamH I

FIGURE 18A

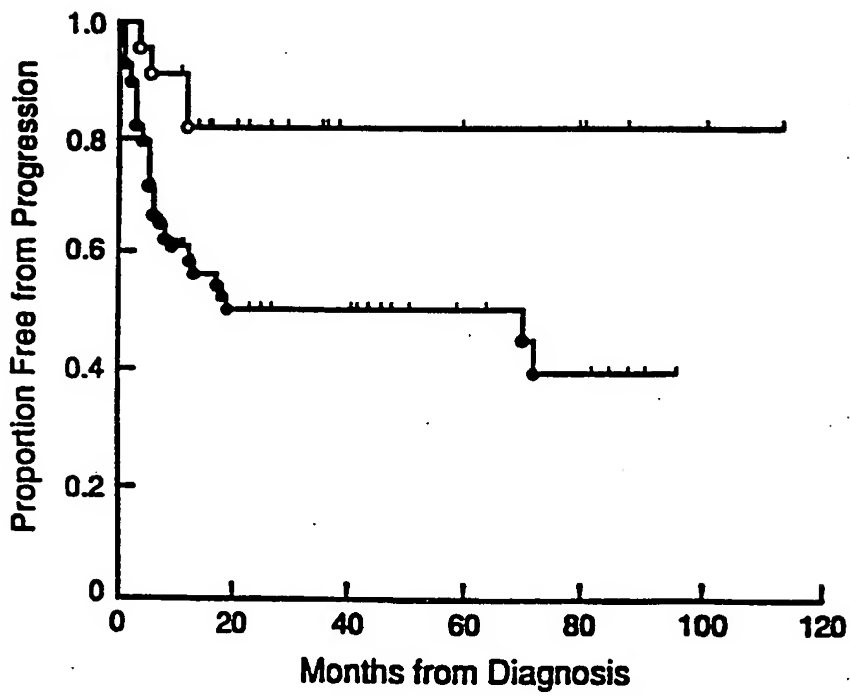

1.0
0.8
0.6
0.4
0.2
0
Proportion Free from Progression
0
20
40
60
80
100
120
Months from Diagnosis

FIGURE 18B

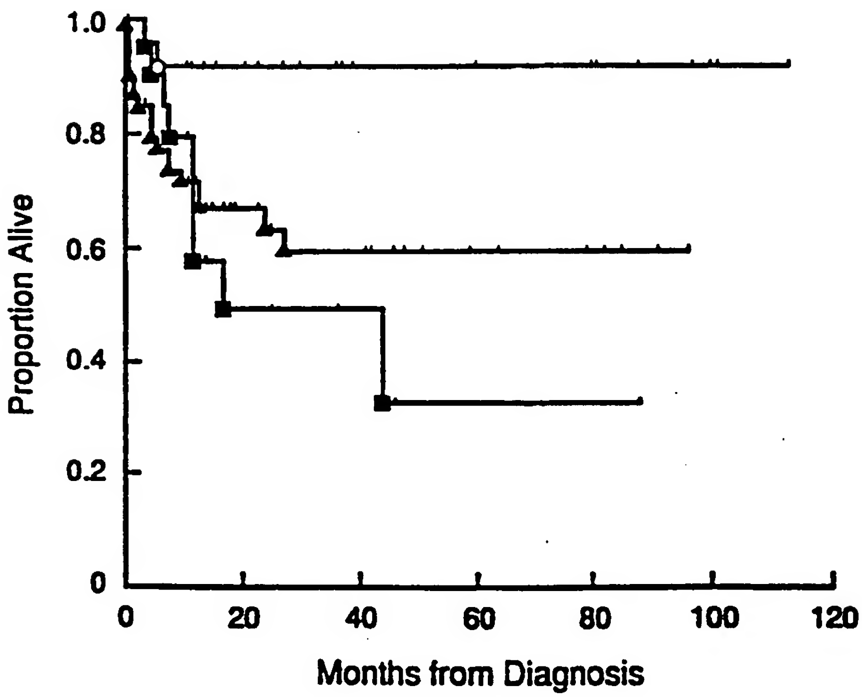

1.0
0.8
0.6
0.4
0.2
0
Proportion Alive
0
20
40
60
80
100
120
Months from Diagnosis

FIGURE 19A

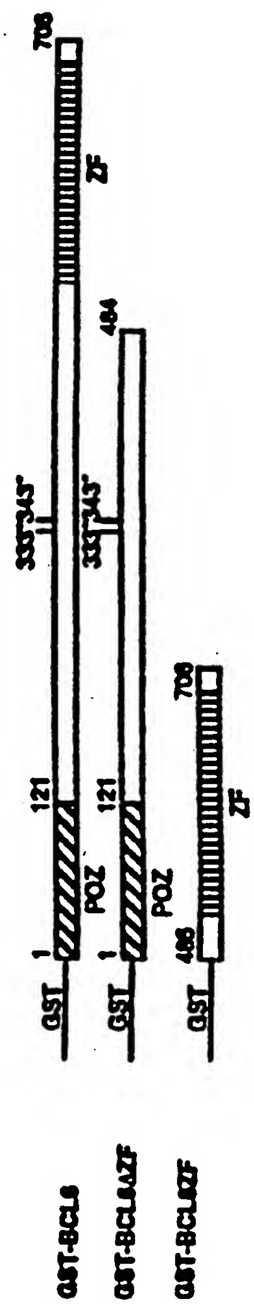

GST-BCL6
GST-BCL6ΔZF
GST-BCL6ZF
GST
1
121
333
343
706
POZ
ZF
484
486

FIGURE 19B

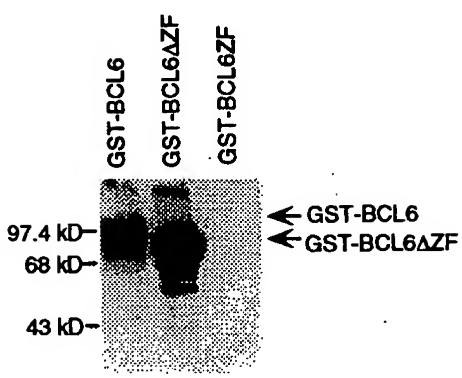


FIGURE 19C

FIGURE 20A

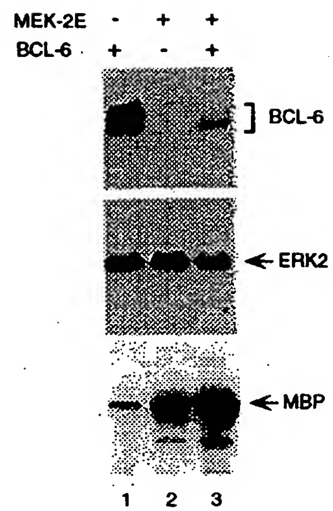


FIGURE 20B

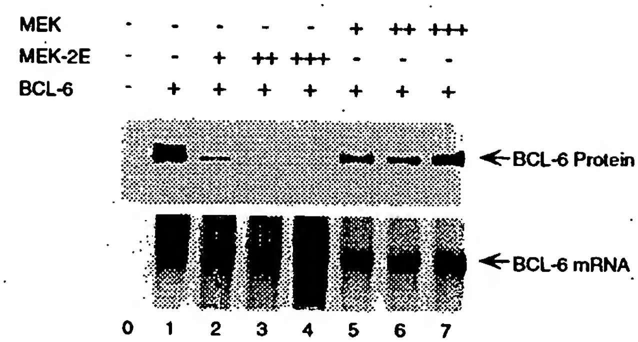


FIGURE 20C

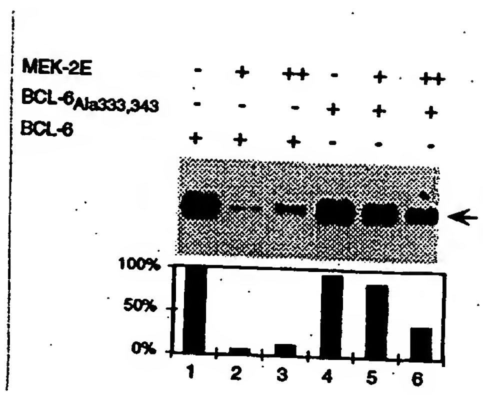


FIGURE 20D

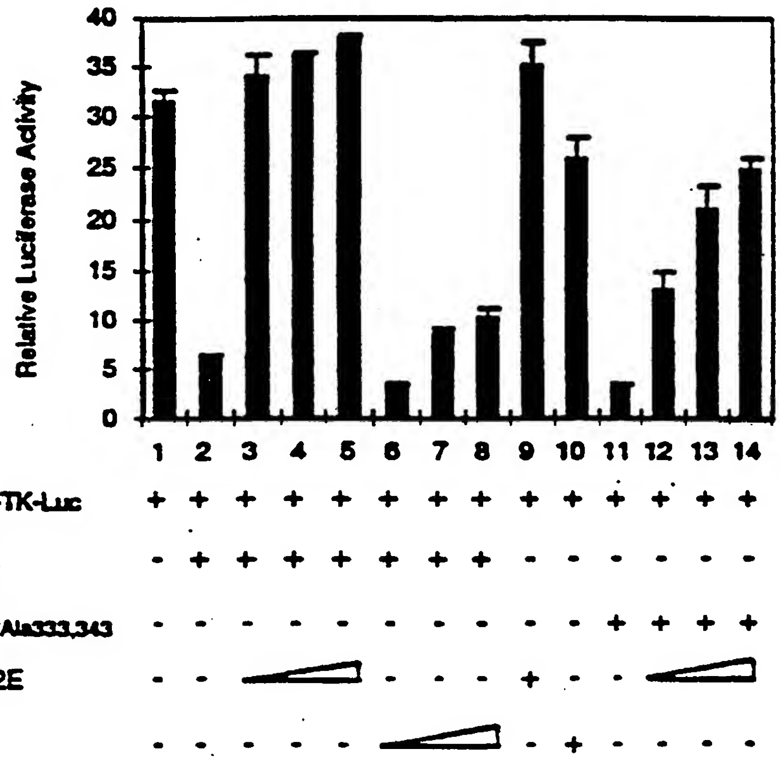

Relative Luciferase Activity
0
5
10
15
20
25
30
35
40
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B6BS-TK-Luc + + + + + + + + + + + + + +
BCL-6 - + + + + + + + - - - - - -
BCL-6Ala333,343 - - - - - - - - - - + + + +
MEK-2E - - - - + - - - +
MEK - - - - - - + - - - -

FIGURE 21A

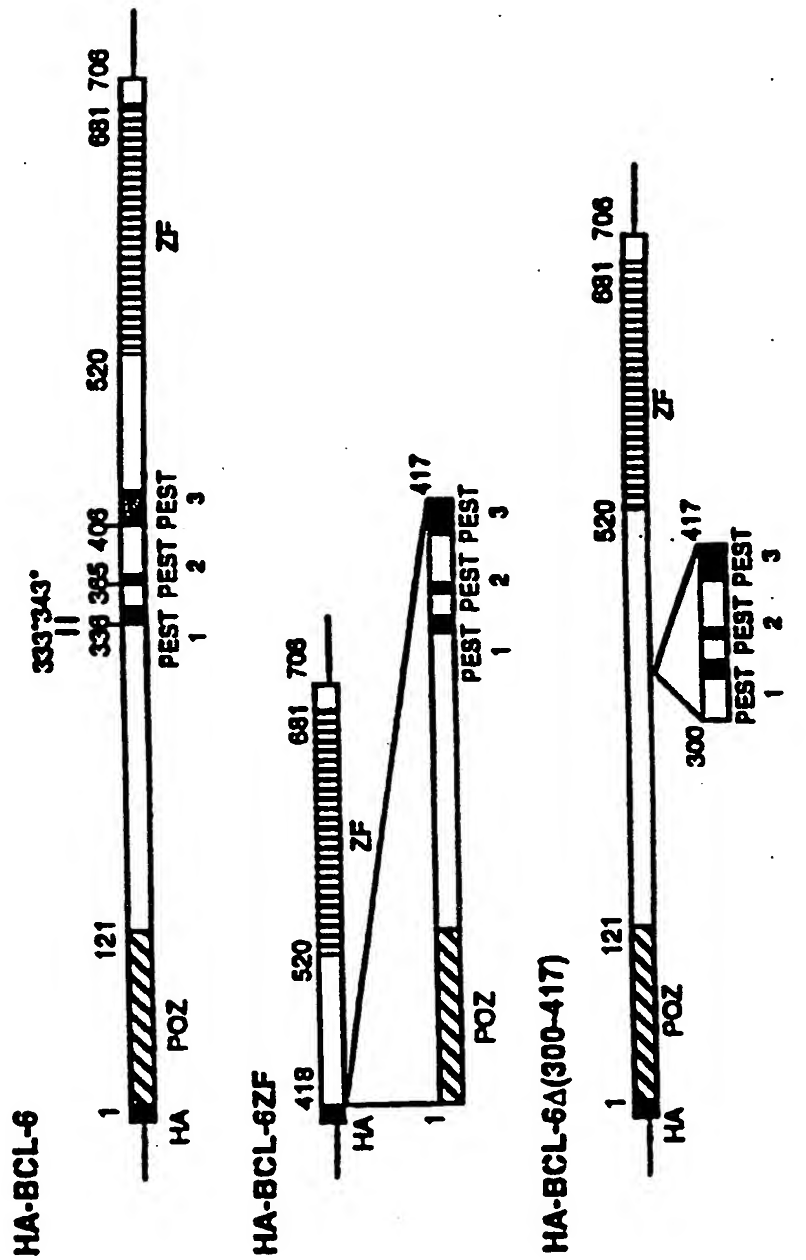

HA-BCL-6
1
121
333
365
406
520
681
706
333~343*
HA
POZ
PEST 1
PEST 2
PEST 3
ZF
HA-BCL-6ZF
418
520
681
706
1
417
HA
ZF
POZ
PEST 1
PEST 2
PEST 3
HA-BCL-6Δ(300-417)
1
121
520
681
706
300
417
HA
POZ
ZF
PEST 1
PEST 2
PEST 3

FIGURE 21B

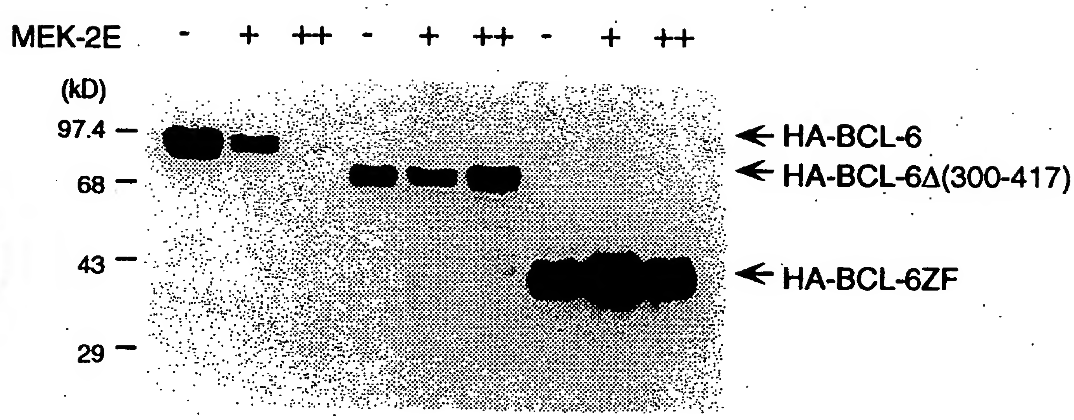


FIGURE 22A

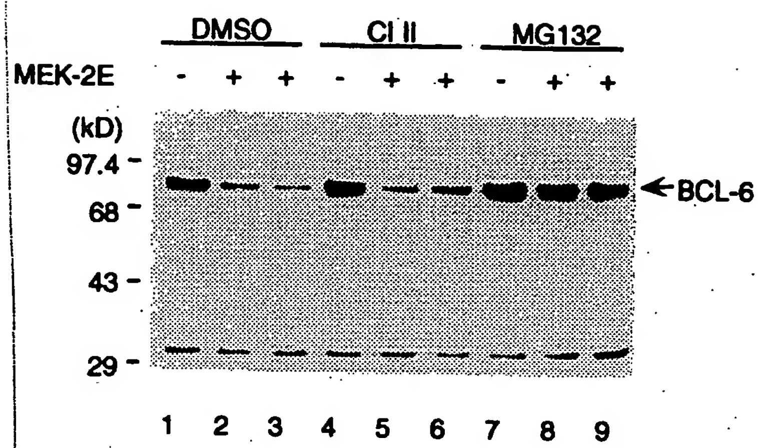


FIGURE 22B

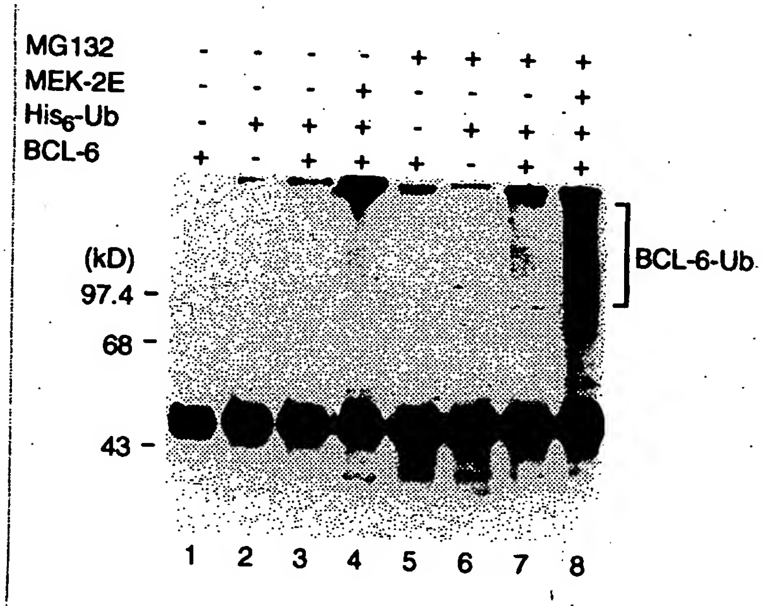


FIGURE 23A

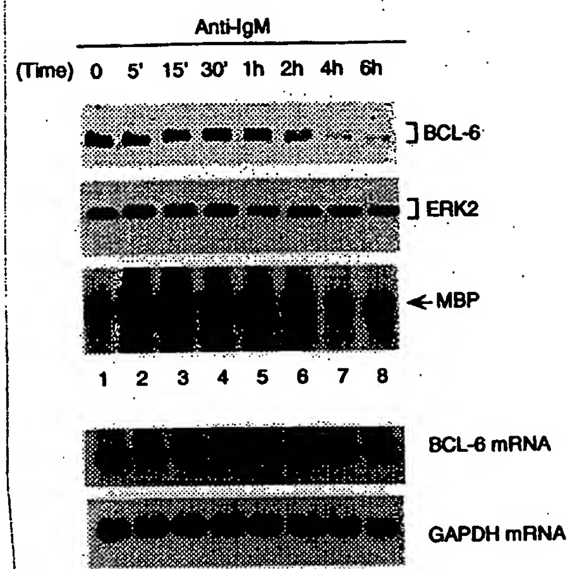


FIGURE 23B

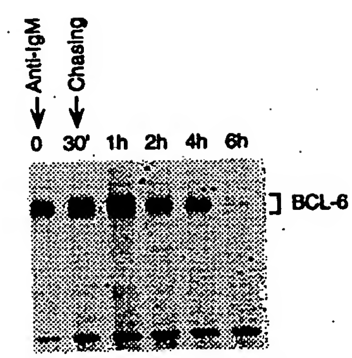


FIGURE 23C

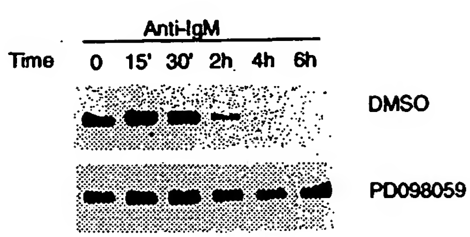


FIGURE 23D

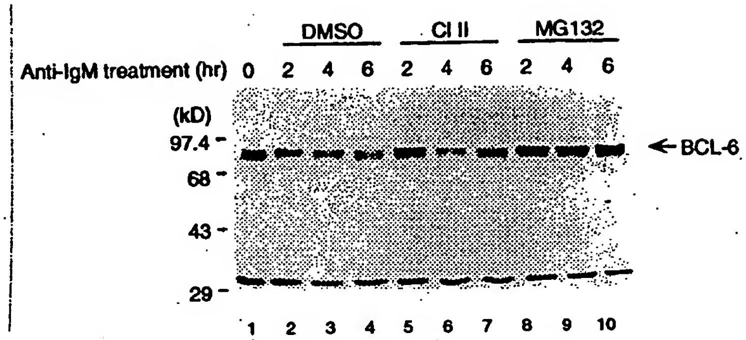
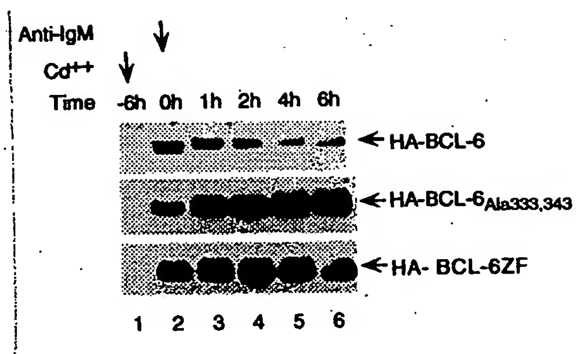


FIGURE 23E



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14703

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/00, 38/19, 38/20, 38/45, 39/395
US CL :428/85.1, 94.1, 130.1; 514/1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.1, 94.1, 130.1; 514/1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	LIN et al. Anti-IgM-induced growth inhibition and apoptosis are independent of ornithine decarboxylase in Ramos cells. Experimental Cell Research. 25 November 1997, Vol. 237, No. 1, pages 231-241, especially pages 231-232.	1, 5-9, 12, 13 — 14-18, 21-23
X — Y	MORIYAMA et al. BCL-6 is phosphorylated at multiple sites in its serine- and proline-clustered region by mitogen activated protein kinase (MAPK) *in vivo*. Oncogene. 22 May 1997, Vol. 14, No. 20, pages 2465-2474, especially page 2465.	1, 2, 4 — 3
X	US 4,863,727 A (ZIMMERMAN et al.) 05 September 1989, see claim 8.	14, 16, 19, 20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 SEPTEMBER 1999	09 November 1999 (09.11.99)

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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DAVID S. ROMEO
Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SEMMEL et al. Differentiation of Burkitt lymphoma cells by hexamethylenbisacetimide. Molecular Biology Reports. 1989, Vol. 13, pages 151-157, especially page 151.	24-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14703

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE
search terms: lymphoma, hodgkin's, anti-IgM, IL-2, IL-6, TNF, HMBA, trichostatin, bcl-6, germinal centers, large cell lymphoma, laz3, bcl5

```
CC   cell lymphoma in a subject. Anti-BCL-6 antibodies may also be used for
CC   this purpose. The methods are useful for treating non-Hodgkin's lymphoma
XX
SQ   Sequence 706 AA;

  Query Match             100.0%;  Score 3793;  DB 3;  Length 706;
  Best Local Similarity   100.0%;  Pred. No. 9.8e-284;
  Matches  706;  Conservative    0;  Mismatches    0;  Indels    0;  Gaps    0;

Qy          1 MASPADSCIQFTRHASDVLLNLNRLRSRDILTDVVIVVSREQFRAHKTVLMACSGLFYSI 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db          1 MASPADSCIQFTRHASDVLLNLNRLRSRDILTDVVIVVSREQFRAHKTVLMACSGLFYSI 60

Qy         61 FTDQLKCNLSVINLDPEINPEGFCILLDFMYTSRLNLREGNIMAVMATAMYLQMEHVVDT 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db         61 FTDQLKCNLSVINLDPEINPEGFCILLDFMYTSRLNLREGNIMAVMATAMYLQMEHVVDT 120

Qy        121 CRKFIKASEAEMVSAIKPPREEFLNSRMLMPQDIMAYRGREVVENNLPLRSAPGCESRAF 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        121 CRKFIKASEAEMVSAIKPPREEFLNSRMLMPQDIMAYRGREVVENNLPLRSAPGCESRAF 180

Qy        181 APSLYSGLSTPPASYSMYSHLPVSSLLFSDEEFRDVRMPVANPFPKERALPCDSARPVPG 240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        181 APSLYSGLSTPPASYSMYSHLPVSSLLFSDEEFRDVRMPVANPFPKERALPCDSARPVPG 240

Qy        241 EYSRPTLEVSPNVCHSNIYSPKETIPEEARSDMHYSVAEGLKPAAPSARNAPYFPCDKAS 300
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Db        241 EYSRPTLEVSPNVCHSNIYSPKETIPEEARSDMHYSVAEGLKPAAPSARNAPYFPCDKAS 300

Qy        301 KEEERPSSEDEIALHFEPPNAPLNRKGLVSPQSPQKSDCQPNSPTEACSSKNACILQASG 360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        301 KEEERPSSEDEIALHFEPPNAPLNRKGLVSPQSPQKSDCQPNSPTEACSSKNACILQASG 360

Qy        361 SPPAKSPTDPKACNWKKYKFIVLNSLNQNAKPGGPEQAELGRLSPRAYTAPPACQPPMEP 420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        361 SPPAKSPTDPKACNWKKYKFIVLNSLNQNAKPGGPEQAELGRLSPRAYTAPPACQPPMEP 420

Qy        421 ENLDLQSPTKLSASGEDSTIPQASRLNNIVNRSMTGSPRSSSESHSPLYMHPPKCTSCGS 480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        421 ENLDLQSPTKLSASGEDSTIPQASRLNNIVNRSMTGSPRSSSESHSPLYMHPPKCTSCGS 480

Qy        481 QSPQHAEMCLHTAGPTFAEEMGETQSEYSDSSCENGAFFCNECDCRFSEEASLKRHTLQT 540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Qy        541 HSDKPYKCDRCQASFRYKGNLASHKTVHTGEKPYRCNICGAQFNRPANLKTHTRIHSGEK 600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Qy        601 PYKCETCGARFVQVAHLRAHVLIHTGEKPYPCEICGTRFRHLQTLKSHLRIHTGEKPYHC 660
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Qy        661 EKCNLHFRHKSQLRLHLRQKHGAITNTKVQYRVSATDLPPELPKAC 706
              ||||||||||||||||||||||||||||||||||||||||||||||
Db        661 EKCNLHFRHKSQLRLHLRQKHGAITNTKVQYRVSATDLPPELPKAC 706

RESULT 2
AAB29640
ID   AAB29640 standard; protein; 706 AA.
XX
AC   AAB29640;
XX
DT   23-FEB-2001  (first entry)
XX
DE   Human bcl-6 transcriptional repressor.
XX
KW   Human; bcl-6; transcriptional repressor; germinal centre formation;
KW   Th-2 mediated antibody affinity maturation; apoptosis regulator;
KW   chromosome 3q27; lymphoma; acute lymphoblastic leukaemia;
KW   post-transplant lymphoproliferative disorder; expression inhibition;
KW   antisense therapy.
XX
OS   Homo sapiens.
XX
PN   US6140125-A.
XX
PD   31-OCT-2000.
XX
PF   15-OCT-1999;   99US-00418640.
XX
PR   15-OCT-1999;   99US-00418640.
XX
PA   (ISIS-) ISIS PHARM INC.
XX
PI   Taylor JK,  Cowsert LM;
XX
DR   WPI; 2001-048959/06.
DR   N-PSDB; AAC81137.
XX
PT   Antisense compounds which specifically hybridize with and inhibit human
PT   bcl-6 expression, useful for treating bcl-6 related disorders, and
PT   preventing or delaying inflammation or tumor formation.
XX
PS   Disclosure; Col 47-52; 42pp; English.
XX
CC   This sequence represents human bcl-6. Bcl-6 (also known as B-cell
CC   CLL/lymphoma 6, zinc finger protein 51 and LAZ3) is a sequence- specific
CC   DNA-binding transcriptional repressor. The bcl-6 gene is expressed in
CC   germinal centre B- and T- cells and is required for germinal centre
CC   formation and Th-2 mediated antibody affinity maturation. Bcl-6 may also
CC   play a role in the regulation of apoptosis. The bcl-6 gene is located on
CC   chromosome 3q27, a region which undergoes a high frequency of
CC   translocation events. Such chromosomal translocations can result in
CC   aberrant forms of bcl-6, which are strongly implicated in the
CC   pathogenesis of several types of lymphoma, and have also been reported in
CC   acute lymphoblastic leukaemia and post-transplant lymphoproliferative
CC   disorders. The invention relates to antisense oligonucleotides targetted
CC   to the human bcl-6 gene, which inhibit its expression. A series of
CC   oligonucleotides (AAC81144-C81223) were designed to target different
CC   regions of the human bcl-6 mRNA, and were analysed for their effect on
CC   bcl-6 mRNA levels by quantitative real-time PCR. The oligonucleotides of
CC   the invention are useful for diagnosis, prevention and treatment of
CC   conditions associated with aberrant forms of bcl-6, such as lymphomas,
CC   acute lymphoblastic leukaemia and post-transplant lymphoproliferative
CC   disorders
XX
SQ   Sequence 706 AA;

  Query Match             100.0%;  Score 3793;  DB 4;  Length 706;
  Best Local Similarity   100.0%;  Pred. No. 9.8e-284;
  Matches  706;  Conservative    0;  Mismatches    0;  Indels    0;  Gaps    0;

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              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db          1 MASPADSCIQFTRHASDVLLNLNRLRSRDILTDVVIVVSREQFRAHKTVLMACSGLFYSI 60

Qy         61 FTDQLKCNLSVINLDPEINPEGFCILLDFMYTSRLNLREGNIMAVMATAMYLQMEHVVDT 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db         61 FTDQLKCNLSVINLDPEINPEGFCILLDFMYTSRLNLREGNIMAVMATAMYLQMEHVVDT 120

Qy        121 CRKFIKASEAEMVSAIKPPREEFLNSRMLMPQDIMAYRGREVVENNLPLRSAPGCESRAF 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        121 CRKFIKASEAEMVSAIKPPREEFLNSRMLMPQDIMAYRGREVVENNLPLRSAPGCESRAF 180

Qy        181 APSLYSGLSTPPASYSMYSHLPVSSLLFSDEEFRDVRMPVANPFPKERALPCDSARPVPG 240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        181 APSLYSGLSTPPASYSMYSHLPVSSLLFSDEEFRDVRMPVANPFPKERALPCDSARPVPG 240

Qy        241 EYSRPTLEVSPNVCHSNIYSPKETIPEEARSDMHYSVAEGLKPAAPSARNAPYFPCDKAS 300
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Db        241 EYSRPTLEVSPNVCHSNIYSPKETIPEEARSDMHYSVAEGLKPAAPSARNAPYFPCDKAS 300

Qy        301 KEEERPSSEDEIALHFEPPNAPLNRKGLVSPQSPQKSDCQPNSPTEACSSKNACILQASG 360
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```

```
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Db        361 SPPAKSPTDPKACNWKKYKFIVLNSLNQNAKPGGPEQAELGRLSPRAYTAPPACQPPMEP 420

Qy        421 ENLDLQSPTKLSASGEDSTIPQASRLNNIVNRSMTGSPRSSSESHSPLYMHPPKCTSCGS 480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        421 ENLDLQSPTKLSASGEDSTIPQASRLNNIVNRSMTGSPRSSSESHSPLYMHPPKCTSCGS 480

Qy        481 QSPQHAEMCLHTAGPTFAEEMGETQSEYSDSSCENGAFFCNECDCRFSEEASLKRHTLQT 540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        481 QSPQHAEMCLHTAGPTFAEEMGETQSEYSDSSCENGAFFCNECDCRFSEEASLKRHTLQT 540

Qy        541 HSDKPYKCDRCQASFRYKGNLASHKTVHTGEKPYRCNICGAQFNRPANLKTHTRIHSGEK 600
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Db        541 HSDKPYKCDRCQASFRYKGNLASHKTVHTGEKPYRCNICGAQFNRPANLKTHTRIHSGEK 600

Qy        601 PYKCETCGARFVQVAHLRAHVLIHTGEKPYPCEICGTRFRHLQTLKSHLRIHTGEKPYHC 660
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Db        601 PYKCETCGARFVQVAHLRAHVLIHTGEKPYPCEICGTRFRHLQTLKSHLRIHTGEKPYHC 660

Qy        661 EKCNLHFRHKSQLRLHLRQKHGAITNTKVQYRVSATDLPPELPKAC 706
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Db        661 EKCNLHFRHKSQLRLHLRQKHGAITNTKVQYRVSATDLPPELPKAC 706
```

RESULT 3
ADL82847

```
ID   ADL82847 standard; protein; 706 AA.
XX
AC   ADL82847;
XX
DT   17-JUN-2004  (first entry)
XX
DE   Human PRO26296, SEQ ID 49.
XX
KW   Immunosuppressive; Cytostatic; Antiarthritic; Antirheumatic; Antianemic;
KW   Antiallergic; Muscular; Neuroprotective; Nephrotropic; Antiinflammatory;
KW   Gene Therapy; PRO; B cell related disorder; cancer;
KW   immune-mediated inflammatory disease; human.
XX
OS   Homo sapiens.
XX
PN   WO2004024097-A2.
XX
PD   25-MAR-2004.
XX
PF   15-SEP-2003; 2003WO-US029097.
XX
PR   16-SEP-2002; 2002US-0411392P.
XX
PA   (GETH ) GENENTECH INC.
XX
PI   Chiu H,  Clark H,  Dennis K,  Fong S,  Schoenfeld JR,  Wood WI;
PI   Wu TD;
XX
DR   WPI; 2004-329389/30.
DR   N-PSDB; ADL82846.
XX
PT   New PRO polypeptide, useful for diagnosing and treating a B cell related
PT   disorder, e.g. Burkitt's lymphoma, rheumatoid arthritis, autoimmune
PT   mediated hemolytic anemia, myasthenia gravis or ankylosing spondylitis.
XX
PS   Claim 10; Fig 49; 695pp; English.
XX
CC   The present invention relates to PRO proteins and their coding sequences.
CC   The PRO proteins are useful for diagnosing and treating a B cell related
CC   disorder, e.g. X-linked infantile hypogammaglobulinemia, polysaccharide
CC   antigen unresponsiveness, selective IgA deficiency, selective IgM
CC   deficiency, selective deficiency of IgG subclasses, immunodeficiency with
CC   hyper IgM, transient hypogammaglobulinemia of infancy, Burkitt's
CC   lymphoma, intermediate lymphoma, follicular lymphoma, type II
CC   hypersensitivity, rheumatoid arthritis, autoimmune mediated haemolytic
CC   anaemia, myasthenia gravis, hypoadrenocorticism, glomerulonephritis, or
CC   ankylosing spondylitis. The PRO proteins are also useful for preparing a
CC   medicament for treating a condition that is responsive to the PRO
CC   protein, e.g. cancer or immune-mediated inflammatory diseases. The PRO
CC   coding sequences are useful as hybridization probes in chromosome and
CC   gene mapping, in preparing PRO proteins, or in generating transgenic
CC   animals or knockout animals, which in turn are useful in the development
CC   and screening of therapeutically useful reagents.
XX
SQ   Sequence 706 AA;

  Query Match             100.0%;  Score 3793;  DB 8;  Length 706;
  Best Local Similarity   100.0%;  Pred. No. 9.8e-284;
  Matches  706;  Conservative    0;  Mismatches    0;  Indels    0;  Gaps    0;

Qy          1 MASPADSCIQFTRHASDVLLNLNRLRSRDILTDVVIVVSREQFRAHKTVLMACSGLFYSI 60
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Db          1 MASPADSCIQFTRHASDVLLNLNRLRSRDILTDVVIVVSREQFRAHKTVLMACSGLFYSI 60

Qy         61 FTDQLKCNLSVINLDPEINPEGFCILLDFMYTSRLNLREGNIMAVMATAMYLQMEHVVDT 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db         61 FTDQLKCNLSVINLDPEINPEGFCILLDFMYTSRLNLREGNIMAVMATAMYLQMEHVVDT 120

Qy        121 CRKFIKASEAEMVSAIKPPREEFLNSRMLMPQDIMAYRGREVVENNLPLRSAPGCESRAF 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        121 CRKFIKASEAEMVSAIKPPREEFLNSRMLMPQDIMAYRGREVVENNLPLRSAPGCESRAF 180

Qy        181 APSLYSGLSTPPASYSMYSHLPVSSLLFSDEEFRDVRMPVANPFPKERALPCDSARPVPG 240
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Db        181 APSLYSGLSTPPASYSMYSHLPVSSLLFSDEEFRDVRMPVANPFPKERALPCDSARPVPG 240

Qy        241 EYSRPTLEVSPNVCHSNIYSPKETIPEEARSDMHYSVAEGLKPAAPSARNAPYFPCDKAS 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        241 EYSRPTLEVSPNVCHSNIYSPKETIPEEARSDMHYSVAEGLKPAAPSARNAPYFPCDKAS 300

Qy        301 KEEERPSSEDEIALHFEPPNAPLNRKGLVSPQSPQKSDCQPNSPTEACSSKNACILQASG 360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        301 KEEERPSSEDEIALHFEPPNAPLNRKGLVSPQSPQKSDCQPNSPTEACSSKNACILQASG 360

Qy        361 SPPAKSPTDPKACNWKKYKFIVLNSLNQNAKPGGPEQAELGRLSPRAYTAPPACQPPMEP 420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        361 SPPAKSPTDPKACNWKKYKFIVLNSLNQNAKPGGPEQAELGRLSPRAYTAPPACQPPMEP 420

Qy        421 ENLDLQSPTKLSASGEDSTIPQASRLNNIVNRSMTGSPRSSSESHSPLYMHPPKCTSCGS 480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        421 ENLDLQSPTKLSASGEDSTIPQASRLNNIVNRSMTGSPRSSSESHSPLYMHPPKCTSCGS 480

Qy        481 QSPQHAEMCLHTAGPTFAEEMGETQSEYSDSSCENGAFFCNECDCRFSEEASLKRHTLQT 540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        481 QSPQHAEMCLHTAGPTFAEEMGETQSEYSDSSCENGAFFCNECDCRFSEEASLKRHTLQT 540

Qy        541 HSDKPYKCDRCQASFRYKGNLASHKTVHTGEKPYRCNICGAQFNRPANLKTHTRIHSGEK 600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        541 HSDKPYKCDRCQASFRYKGNLASHKTVHTGEKPYRCNICGAQFNRPANLKTHTRIHSGEK 600

Qy        601 PYKCETCGARFVQVAHLRAHVLIHTGEKPYPCEICGTRFRHLQTLKSHLRIHTGEKPYHC 660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        601 PYKCETCGARFVQVAHLRAHVLIHTGEKPYPCEICGTRFRHLQTLKSHLRIHTGEKPYHC 660

Qy        661 EKCNLHFRHKSQLRLHLRQKHGAITNTKVQYRVSATDLPPELPKAC 706
              ||||||||||||||||||||||||||||||||||||||||||||||
Db        661 EKCNLHFRHKSQLRLHLRQKHGAITNTKVQYRVSATDLPPELPKAC 706
```

RESULT 4
ADR14017

```
ID   ADR14017 standard; protein; 706 AA.
XX
AC   ADR14017;
XX
DT   21-OCT-2004  (first entry)
XX
```